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The miR-290-295 cluster promotes pluripotency maintenance by regulating cell cycle phase distribution in mouse embryonic stem cells

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ABSTRACT

The mmu-miR-290-295 cluster codes for a family of microRNAs (miRNAs) that are expressed *de novo* during early embryogenesis and are specific for mouse embryonic stem cells (ESC) and embryonic carcinoma cells (ECC). Detailed sequence analysis and alignment studies of miR-290-295 precursors demonstrated that the cluster has evolved by repeated duplication events of the ancient miR-290 precursor. We show that under serum starvation, overexpression of miR-290-295 miRNAs withhold ES cells from early differentiation, ensures their high proliferation rate and capacity for forming alkaline phosphate positive colonies. Transcriptome analysis revealed that differentiation related marker genes are underexpressed upon high miR-290-295 level. Importantly, miR-290-295 overexpression prevents ES cells from accumulation in G1 phase at low serum level, and seems to regulate cell cycle in different phases. Our data underline that miR-290-295 miRNAs contribute to the natural absence of G1 checkpoint in embryonic stem cells. We define the cell cycle regulators Wee1 and Fbxl5 as potential direct targets of miR-290-295 miRNAs in vitro. Our results suggest that miR-290-295 miRNAs exhibit their effect predominantly through the regulation of cell cycle phase distribution.

1. Introduction

Embryonic stem cells (ES cells or ESC) are derived from the inner cell mass of the blastocyst. ES cells can be propagated in vitro without the loss of pluripotency and induced to differentiate into specialized cell types when given appropriate cues, making them potential sources of tissue in regenerative therapies (Mayhall et al., 2004).

The microRNAs (miRNAs) are 20–25 nucleotide (nt) long non-coding RNAs that have been identified as novel regulators of gene expression. They negatively regulate genes by binding to specific sequences in the 3′ UTR of target mRNAs (Lai, 2002) and inhibiting their translation (He and Hannon, 2004) and stability. MiRNAs act in a range of biological processes, including cancer (Dalmay and Edwards, 2006; Zhang et al., 2007), implantation (Chakrabarty et al., 2007), development of the limb (Harfe et al., 2005; Hornstein et al., 2005), lung (Harris et al., 2006) and haematopoietic system (Chen et al., 2004).

The miRNA pathway has been implicated in maintaining stem cell character in both plants (Williams and Fletcher, 2005; Zhao et al., 2007) and animals (Cao et al., 2008; Gu et al., 2008; Marson et al., 2008). Many attempts were made to find ESC specific miRNAs (Hayes et al., 2008; Houbaviy et al., 2003; Suh et al., 2004) to deduce the role of miRNA pathway in mammalian stem cells (Sinkkonen et al., 2008) and during the events of early differentiation (Bibikova et al., 2008; Chen et al., 2007; Hatfield and Ruohola-Baker, 2008; Ivey et al., 2008). Earlier studies focused on dissecting the miRNA pathway through creating targeted gene inactivations and mutations. Dicer homozygous embryos die during gastrulation before the body plan is configured. Oct4 staining of the Dicer mutant embryos was reduced, indicating loss of detectable multipotent stem cells (Bernstein et al., 2003). Dicer mutant ESC lines showed pronounced proliferation defect, slight increase of G0 and G1 cells (Murchison et al., 2005) and failure of teratoma and chimera formation. Embryoid body formation of mutant ESCs showed little morphological evidence of differentiation and expression of differentiation markers (Hnf4, Brachyury (T), Bmp4, Gata1) were compromised (Kanellopoulou et al., 2005). To dissect miRNA pathway from other potential functions of Dicer, DGCR8 knockout ESCs were generated and were shown to arrest early in

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development (Sohn et al., 2007). DGCR8 knockout ESCs looked morphologically normal and expressed ES cell specific markers, but had extended population doubling time. Knockout ES cells were accumulated in the G1 phase of the cell cycle, showing that miRNAs are required for normal ES cell proliferation and cell-cycle progress. Similarly to Dicer knock-out ESCs upon LIF withdrawal or during differentiation with retinoic acid, DGCR8 knockout ES cells could not efficiently silence the ES cell program and start normal differentiation in teratomas (Wang et al., 2007).

The majority of known miRNAs sequenced from mouse ES cell lines could be accounted for six genomic loci, including the most abundant ESC specific miRNAs belonging to mmu-miR-290-295 cluster (miR-290-295) (Calabrese et al., 2007). The members of this miRNA cluster, bearing the AAAGUGC seed, are the functionally dominant miRNAs in mouse ES cells (Sinkkonen et al., 2008).

The miR-290-295 cluster is only present in placental mammals. In the mouse genome, seven related pre-miRNAs (miR-290-miR-295) mapped in the same relative orientation within 2.2 kb region of the genomic sequence, suggesting that this cluster is initially synthesized as a common primary transcript (Houbaviy et al., 2005). The miR-290-295 cluster was shown to be conserved among human, chimpanzee, rat, mouse, dog and cow, although its structure is highly variable (Houbaviy et al., 2005). In mouse it is transcribed as a single, 3.2 kb long pre-miRNA processed into 10 different miRNAs. De novo expression of these miRNAs occurs as early as between two- and four-cell-stages, thus being among the first and most abundant miRNAs expressed during early mouse embryonic development (Tang et al., 2007).

Despite the detailed investigation of miRNA pathway on stem cell behaviour, only few studies targeted the function of individual stem cell-specific miRNAs (Ivanovska et al., 2008; Wang et al., 2008) and the exact function and biologically relevant action of miR-290-295 cluster needs to be explored.

2. Materials and methods

2.1. Sequence analysis

For analysis of sequence similarities Repeat Masker (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) and Tandem Repeats Finder software (http://tandem.bu.edu/trf/trf.advanced.submit.html) were used to mask tandem repetitive elements, while sequence comparison among the miRNA precursors has been carried out by Pipmaker (http://pipmaker.bx.psu.edu/pipmaker/) Alibee (http://www.genebee.msu.su/services/malign_reduced.html), ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and NCBI/BLAST2 (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&SHOW_DEFAULTS=on&BLAST_SPEC=blast2seq&LINK_LOC=align2seq) with the option of 'somewhat similar sequences'. Sequences were obtained from MirBase (http://microrna.sanger.ac.uk/) and Ensemble database (http://www.ensembl.org/index.htm).

2.2. ES cell culture

R1 (Nagy et al., 1993) ES cells were maintained on a feeder layer of embryonic mouse fibroblasts, in ES cell cultivation medium composed of Dulbecco's modified Eagle's medium (KO-DMEM medium; Invitrogen, Carlsbad, CA) supplemented with glutamax (Invitrogen, 100 ×), 50 μg/ml streptomycin (Sigma), 50 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 mM β-mercaptoethanol (Sigma), 0.1 mM non-essential amino acids (Invitrogen), 1000 units/ml of leukemia inhibitory factor (ESGRO) and 15% FCS (HyClone, Logan, UT). Two days before the analysis (proliferation test, in vitro differentiation, colony assay, luciferase assay and sample collection for RNA isolation) ES cells were plated onto 0.1% gelatin (Sigma) coated plates and were cultured in 15% or 2% FCS (HyClone) containing ES cell cultivation medium.

2.3. In vitro differentiation

In vitro differentiation of ES cells to neurons was promoted with retinoic acid (RA), using modified 4−/4+ induction protocol (Bain, 1995). Before neuronal induction, ES cells were transferred to 0.1% gelatin (Sigma) coated dishes (Greiner bio-one, Kremsmuenster, Austria) for 24 h. R1 cells ($n=200$) were cultured as EBs (embryoid bodies) in hanging drops (day 0 of differentiation) for two days, and then were transferred to bacteriological Petri dishes containing differentiation medium (KO-DMEM supplemented with glutamax, 50 μg/ml streptomycin, 50 U/ml penicillin, 50 mM β-mercaptoethanol, 0.1 mM non-essential amino acids and 10% FCS). EBs were transferred into fresh dishes with fresh medium, every second day. All-trans-retinoic acid (10^{-6} M; Sigma) was added for 4 days, between 4th and 8th days. On the 8th day of differentiation, EBs were plated onto gelatin-coated plates. Two sets of experimental cultures were used for this study.

2.4. Immunofluorescence analysis

Cells grown on glass coverslips were washed with PBS, first at room temperature. Fixation was carried out with 4% of paraformaldehyde (Sigma) for 10 min on r.t. and the ES cells were then washed in PBS containing 0.5% BSA (Sigma). For blocking, PBS containing 1% BSA was used for 30 min. Samples were incubated at 4 °C with the primary antibodies overnight and secondary antibodies were added during 1 h at room temperature. Nanog (AF2729, 1:10, goat IgG), Brachyury (AF2085, 1:10, goat IgG), Oct4 (AF1759, 1:10, goat IgG) from R&D Systems, at a concentration of 1 mg/100 ml. Cy3 conjugated anti-goat IgG (in 1:400 dilution, from Jackson ImmunoResearch, USA) were used as secondary antibodies. Fbxl5 antibody was purchased from Santa Cruz (Fbl5 (N-17), sc-54364, 1:50, goat IgG) and has been used as directed by the manufacturer. Nuclear staining was performed by embedding the cells in DAPI containing Vectashield mounting medium (Vector Labs, Burlingame, CA). Samples were investigated under the fluorescence microscope (Nikon, Germany, AxioVision, Zeiss, Germany). Immunostaining has been quantified by AxioVision 4.7.2 software. Only colonies with visible staining have been used for FBXL5 quantification, thus the following colonies were excluded and are only shown on Supplementary Fig. 6: control B ESC line-number 10 colony, #2 ESC line-number 3, 8 and 10 colonies, #3-number 7, 10 and 12 colonies.

2.5. Western blot analysis

Mouse ES cells were grown on 6 cm plates. Cells were scraped, washed in PBS. Centrifuged ESCs were lysed in RIPA buffer (sc-24948, Santa Cruz), including protease inhibitor. Cell lysates were cleared and protein concentrations were determined. Cell free extract (25 μg) was loaded per lane on 8% SDS-PAGE gel. Protein was then transferred to PVDF membrane at 80 V for an overnight. Membranes were blocked in Blotto A (Santa Cruz) with 0.1% tween 20 and then incubated with anti-FBXL5 (FBL5 (H-300) Santa Cruz, 1:100, in rabbit, sc-134984), or anti-GAPDH (Anti-GAPDH antibody produced in rabbit, G9545, SIGMA, 1:5000) for 1 h. Membranes were washed three times for 10 min each prior to incubation in horse radish peroxidase (HRP)-conjugated