SZENT ISTVÁN UNIVERSITY
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Animal Breeding Science Doctoral School

INVESTIGATION OF THE MOUSE EMBRYONIC STEM CELL IN VITRO DIFFERENTIATION AS A MODEL SYSTEM OF CELL DIFFERENTATION

PHD THESIS

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1. INTRODUCTION

The miRNA pathway has been implicated in maintaining stem cell character in both plants and animals. Many attempts were made to find ESC specific miRNAs to deduce the role of miRNA pathway in mammalian stem cells and during the events of early differentiation. 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. 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). The members of this miRNA cluster, bearing the AAAGUGC seed, are the functionally dominant miRNAs in mouse ES cells. The miR-290-295 cluster is only present in placental mammals. In the mouse genome, seven related pre-miRNAs (miR-290- miR295) 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. The miR-290-295 cluster was shown to be conserved among human, chimpanzee, rat, mouse, dog and cow, although its structure is highly variable. 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. Despite the detailed investigation of miRNA pathway on stem cell behaviour, only few studies targeted the function of individual stem cell-specific miRNAs and the exact function and biologically relevant action of miR-290-295 cluster needs to be explored.

2. GOALS

1. **How could the miR-290-295 cluster evolve?**
2. **How does miR-290-295 cluster affect the appearance and growth characteristics of the mouse ES cells?**
3. **How does the overexpression of miR-290-295 cluster alter the transciptome of the mouse ES cells?**
4. **Can we define a main biological process/pathway through which miR-290-295 regulates mouse ES cells?**
5. **Does miR-290-295 affect the differentiation abilities of mouse ES cells?**

3. RESULTS AND CONCLUSIONS

The mouse miR-290-295 cluster has evolved by multiple duplication events.

Mouse miR-290-295 cluster consists of seven miRNA precursors: miR-290, miR-291a, miR-292, miR-291b, miR-293, miR-294 and miR-295, downstream of Nlrp12 gene. Similar location is mapped for its known human homologues: miR-371, miR-372 and miR-373 (miR-371-373 cluster). To assess the evolutionary relation among miR-290-295 cluster members, we first searched for duplications in the 20 kb vicinity of miR-290-295 coding genomic sequence. A repeat masked sequence was subjected to the Tandem Repeats Finder software and was also blasted to itself, in order to find repeated sequences. Both approaches revealed the presence of a truncated repeat overlapping with miR-290-295. The first repeat consists of miR-290, miR- 291a, miR-292 and miR-291b, while the truncated repeat harbors miR-293, miR-294 and miR-295. We next looked for similarities among miR-290-295 cluster members to understand the duplication events in mouse. Alignment programs (NCBI Blast2, Alibee and ClustalW) distinguished two closely related groups: miR-290, miR-292, miR-293, miR-295 and miR-291a, miR- 291b, miR-294. Taken together the results of the repeat search and sequence alignments, we speculate that miR-290 and/or 291a could be the ancestor of miR-290-295 cluster. By duplication they generated miR-292 and 291b, and later the stretch of these four miRNA precursors has partially duplicated and established miR-293, miR-294 and miR-295. This hypothesis implies that miR-290 and/or miR-291a are/is the ancestor of the cluster, and therefore should be present in other

species. To this aim, we compared the mouse miR-290-295 sequence to its human homologue, miR-371- 373 cluster (including the neighboring 20 kb regions). A single 74 bp long sequence showed high conservation between the two species, and proved to be the mouse miR-290 or the human miR-371 precursor itself. miR-291a did not show similarity to miR- 371-373 region.

miR-290-295 cluster affects colony formation and proliferation of murine ES cells.

To study the developmental function of mmu-miR-290-295 cluster, miRNA coding region of the entire miR-290-295 cluster was cloned to pEF6/V5-His TOPO TA vector (Invitrogen) under the regulation of the constitutive EF-1a promoter. Stable mouse ES cell lines, expressing the whole miR-290-295 cluster were generated. miR-290 expression of ES clones #1, #2 and #3 was measured by quantitative RT-PCT and showed 1.71, 1.93 and 2.37 fold overexpression (respectively), compared to control ESC lines (transfected by empty cloning vector or PBS). The miR-290-295 overexpressing ESC lines were morphologically normal, stained alkaline phosphate (AP) positive and were positive for all examined stemness markers. Colony formation ability of control and miR-290-295 overexpressing ESC lines was assessed and AP+ colonies were counted. Since ESCs exhibit high endogenous level of miR-290-295, LIF withdrawal and low serum conditions were tested to reveal the possible differences between control and overexpressing cell line character. In the absence of LIF, #1, #2 and #3 cell lines showed higher number of AP+ colonies (15.3, 17.4 and 34.5) than the average of control ESC vector1, vector 2 and A (10.6). However, no difference could be detected in the presence of LIF. Similarly, colony formation of A, B, #2 and #3 lines did not show significant difference when the medium was supplied with 15% FCS; however, upon serum starvation (media containing 2% FCS) miR-290-295 overexpressing ESC lines demonstrate remarkably higher ability for colony formation. In case of #2 and #3 transgenic ESC lines 89.9% and 89.4% of the colonies stained positive for alkaline phosphatase, respectively, while control A and B lines showed only 74% and 63% alkaline phosphatase positive colonies. To test the effect of miR-290-295 on proliferation, #2 and #3 ESC lines were compared with control A and B ESC lines. We did not observe significant difference in the proliferation rate, when ES cells were kept in media supplemented with 15% FCS. However, upon serum starvation, the proliferation rate of #2 and #3 ESC lines was higher (1.54 and 1.39), while the proliferation rate of control A and B ESCs measured 1.11 and 1.07, respectively.

Overexpression of miR-290-295 cluster inhibits the expression of early differentiation markers.

To determine the downstream effects of miR-290-295 overexpression, transcriptome analysis has been performed with the Taqman Low-density Array (TLDA, Applied Biosystem) on Mouse Stem Cell Pluripotency Panel enabling us the simultaneous real-time qRT-PCR based quantification of 96 stemness and differentiation related mRNA transcripts. The examined genes were grouped into two major clusters. 'Cluster 1' genes show elevated expression in the control ESC lines (A, B and C) compared to the miR-290-295 overexpressing #2 and #3 ESC lines. 'Cluster 1' contains several early differentiation markers: Actc1, Desmin, Fgf5 (endoderm differentiation), Brachyury (T), Flt1 (mesoderm differentiation) and Gal, Noggin, Nestin (neuroectoderm, neural progenitors) along with the trophoblast marker Cdx2 and Eomes. 'Cluster 2' genes did not show differential expression between the miR-290-295 overexpressing and control samples or the changes were minor and visually not significant. This cluster mainly contains of stemness markers (Pou5f1, Sox2 and Fgf4, FoxD3, Lin28, Cd9, Utf1, Zfp42 and Lifr). Immunohistochemistry has been performed to confirm the results of TLDA analysis at protein level. Nanog was equally expressed in both the control and miR-290-295 overexpressing ESC clones. In accordance with the RNA expression analysis, Brachyury (T), an early mesodermal differentiation marker protein, showed reduced protein level in miR-290-295 overexpressing #2 and #3 ES cells compared to control C ES cell line.

The mouse miR-290-295 cluster regulates cell cycle at multiple points.

To assess the function and the relevance of miR-290-295 cluster in cell cycle regulation, cell cycle profiles of miR-290-295 overexpressing clones (#1, #2 and #3), and control clones (vector 1 and A ESC lines) were determined by staining with propidium iodide and subsequent cell sorting. When serum content was decreased to 2%, the miR-290-295 overexpressing line #3 showed a significant 8% increase of S-phase cells, along with 5% decrease of G0/G1- and 4% decrease of G2/M-phase cells. These data indicate that the miR-290-295 cluster regulates ESC cell cycle at multiple points. Besides the blocking of G1-S transition checkpoint, it might enhance ES cells to enter mitosis, thus speeding up the cell cycle. Wang et al. demonstrated an approximate 5% increase of G1/G0-phase cells, 6% decrease of S-phase cells and 4% increase of G2/M-phase cells in the DGCR8 knockout mouse ES cell model (Wang et al., 2007). Our analysis shows similar extent of changes in cell cycle phase distribution, indicating that the effect of miRNA pathway on the ESC cell cycle is predominantly due to the increased level of the miR-290-295 cluster.

Fbxl5 and Wee1 are potential targets of miR-290-295 cluster members.

In order to identify in silico, the targets of mmu-miR-290-295 cluster, PicTar and miRBase programs were used. Numerous cell cycle regulators were predicted as potential targets (Fbxl5, Skp2, Wee1, p21, Ccng2, Plk1), thus we only considered the mRNAs that were predicted to be targeted by multiple members of miR-290- 295 cluster and the target sites showed conservation between human, rat and mouse. Two cell cycle regulators and a translation elongation factor were chosen for an in vitro confirmation of direct regulation by miR-290-295 cluster. Fbxl5 gene encodes a member of the F-box protein family that constitutes one of the four subunits of the ubiquitin protein ligase complex SCFs (SKP1-cullin-F-box). Fbxl5 interacts with dynactin-1 and orchestrates its turnover via ubiquitination. Destabilization of the centrosomal pool of dynactin causes abnormal G1 centriole separation and delayed entry into an S phase. The correct balance of centrosome- associated dynactin ultimately governs the G1/S transition. Wee1 kinase regulates the G2/M cell cycle checkpoint by phosphorylating and inactivating the mitotic cyclin-dependent kinase 1 (Cdk1). Loss of Wee1 in many systems, including yeast and drosophila, leads to premature mitotic entry.

To test the possibility of direct interaction between Eef1b2, Fbxl5 and Wee1 mRNAs and mmu-miR-290-295 cluster derived miRNAs, we integrated the full-length 3'UTRs of the candidate genes into Renilla luciferase coding reporter vector. Fibroblast, control B and #2 over- expressing ES cell lines were used for the transfection assays. The activities of Renilla and Photinus luciferase were measured by Dual-Luciferase Assay Kit (Promega), Photinus luciferase activity served as a control for transfection efficiency. Fibroblast cells do not express miR-290-295 miRNAs, therefore, were used as negative control for the luciferase studies. The ratio of Renilla and Photinus luciferase activity measured in B and #2 were normalized against Renilla:Photinus ratios of fibroblast cells. ES cells highly express the miR-290-295 miRNA cluster; however, cotransfection of RL-Eef1b2 and PL vector into the control B ES cell line did not change Renilla:Photinus ratio (1.1) compared to that of fibroblast cells. We concluded that Eef1b2 is not directly targeted by miR-290-295 cluster in mouse ES cells. On the contrary, relative Renilla luciferase activity turned remarkably low (0.1) when RL-Fbxl5 vector was introduced. Transfection into the miR-290-295 overexpressing #2 cell line resulted in further decrease (to 0.03) of Renilla luciferase activity. Using RL-Wee1 vector, transfection of control ES cells decreased the Renilla luciferase activity to 0.39 and transfection of #2 cells showed that an activity of Renilla luciferase dropped to 0.11. Decreased activity of Renilla luciferase may result

from the translational inhibition or the destabilization of luciferase mRNA. To dissect these two possibilities, we quantified the Renilla luciferase mRNA level in fibroblast, control B, #2 ESC lines, cotransfected by PL and hRL-TK, RL-Fbxl5 or RL-Wee1 vectors. qRT-PCR analysis failed to reveal any difference in luciferase expression, implying that the cause of decreased Renilla luciferase activity is posttransciptional. To test the specificity of the interaction between miR-290-295 and Fbxl5 and Wee1 3'UTRs, we repeated the dual-luciferase assays with RL constructs carrying the reverse 3'UTRs of the predicted targets (Eef1b2 3'UTRrev, Fbxl5 3'UTRrev and Wee1 3'UTRrev vectors). None of the reverse 3'UTRs was able to compromise Renilla luciferase activity. Renilla:Photinus ratios measured 1.64 for Eef1b2 3'UTRrev, 1.1 for Fbxl5 3'UTRrev and 2.26 for Wee1 3'UTRrev.

To further prove the direct relation between miR-290-295 cluster and the predicted targets, we examined the effect of miR-290-295 cluster at protein level. Fbxl5 is highly expressed both in fibroblast and pluripotent ES cells. Since Wee1 is absent in ES cells and is only expressed during differentiation, we only included Fbxl5 in the protein level studies. Immunostaining showed high protein level of FBXL5 in control B ES cells. We could detect only a minor decrease of FBXL5 protein expression in miR-290-295 overexpressing lines, however, under serum starvation (2% FCS) only low level of defined staining was observed in #2 or #3 cells. Similarly, quantification of Western blot analysis reveals that FBXL5 protein level is deteriorated in #2 and #3 transgenic lines when compared to A and B control ESCs (band densities measured B ESC line is 1.31, while the values are 0.77 in #2 and 0.33 in #3 transgenic lines. FBXL5 protein measures 0.53 in CD1 fibroblast).

Members of miR-290-295 cluster can delay, but are unable to inhibit the differentiation of mouse ES cells.

To have a closer insight into miR-290-295 miRNA function during differentiation, first we tested the effect of miR-290-295 overexpession upon further passages on gelatin-coated plates. We also compared the result of prolonged serum starvation conditions and RA treatment on control and transgenic ESC lines. Passing ES cells without feeder cells on gelatin-coated surface resulted in highly elevated miR-290-295 expression in the transgenic ESC lines. qRT-PCR analysis confirmed increased expression of Oct4 in #2 and #3 ESC lines (0.86 and 1.2) compared to B control line (0.6), along with higher level of Nanog (0.7 and 0.9 compared to 0.4 in B ESCs) and Zfp42 (1.0 and 1.1 compared to 0.4 in control B ESCs). The early differentiation marker Kdr1 expression proved to be lower in the miR-290-295 over- expressing lines (0.9 and 1.0 compared

to 1.9 in B ESC line). Among the differentiation markers, TLDA analysis indicated Brachyury to show the greatest decrease in #2 and #3 ESCs. Increasing passage number by two has a major effect on Brachyury expression. B control ESC line exhibited 20 fold higher mRNA level than #3 and 13.7 fold expression compared to #2 transgenic ESC lines (Fig. 7B). We concluded that miR-290-295 expression augmented the maintenance of high Oct4, Nanog and Zfp42 level when ES cells were deprived from feeder cells, indicating its contribution to preserve pluripotency.

Control A and B ESC lines and #2, #3 miR-290-295 over- expressing lines were propagated under prolonged low serum and normal serum conditions (2% FCS and 15% FCS). Colonies were stained for AP. Control ESC lines grown in media supplemented with 15% FCS showed flattened and differentiating colonies, while miR-290-295 overexpressing ESC lines exhibited normal ES-like colony morphology. However, under long serum starvation (five days) on gelatin-coated dishes, miR-290-295 overexpressing ES lines also show extensive differentiation, indicating that the miR-290-295 overexpression can no longer withdraw differentiation events.

To evaluate the effect of miR-290-295 overexpression in the commitment to a specific (ectodermal) lineage, control B, #2 and #3 ES cell lines were differentiated in the presence of retinoic acid (RA) for nine days. To monitor pluripotency, Oct4 level was measured by qRT-PCR on the fifth and ninth day of RA treatment. On the fifth day, Oct4 expression was 1.75 and 2.65 fold higher in #2 and #3 ESC lines than in control B ES cells. In accordance with our previous results, miR-290-295 overexpres- sion assisted the maintenance of high stem-cell marker expres-sion. However, by the ninth day of RA treatment, all three investigated ESC lines exhibited a remarkable decrease in Oct4 expression, indicating the loss of pluripotency. These results indicate that an increased expression of miR- 290-295, at the level presented in #3 transgenic ESC line, can delay but cannot prevent differentiation of ES cells.

In summary, based on our in vitro results, we suggest that the highly repetitive miR-290-295 cluster might have been evolved to maintain the pluripotency of the inner cell mass for a limited and defined time, and the cluster possibly acts through the direct inhibition of cell cycle phase regulators.

4. NEW SCIENTIFIC RESULTS

1. **Overexpression of the murine miR-290-295 cluster in mouse ES cells inhibits their response for differentiation inducing environment:**
   a. **Aids colony formation and proliferation**
   b. **Decreases the expression of early differentiation markers**
   c. **Contributes to the maintenance of the cell cycle phase distribution that is characteristic to ES cells.**
2. **Fbxl5 is a likely in vivo target of miR-290-295 miRNAs in mouse ES cells.**
3. **Overexpression off miR-290-295 cluster can withhold but is unable to prevent the differentiation of mouse ES cells.**

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