miR-302b maintains “stemness” of human embryonal carcinoma cells by post-transcriptional regulation of Cyclin D2 expression

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ABSTRACT
Embryonic stem cells (ESCs) and embryonal carcinoma cells (ECCs) possess the remarkable property of self-renewal and differentiation potency. They are model preparations for investigating the underlying mechanisms of “stemness”. microRNAs are recently discovered small noncoding RNAs with a broad spectrum of functions, especially in control of development. Here, we show that miR-302b indirectly regulates expression of the pluripotent stem cell marker Oct4, and it directly regulates expression of Cyclin D2 protein, a developmental regulator during gastrulation. Using loss-of function and gain-of function approaches, we demonstrate that functional miR-302b is necessary to maintain stem cell self-renewal and inhibit neuronal differentiation of human ECCs. During retinoic acid-induced neuronal differentiation, Cyclin D2 protein but not mRNA expression is strongly increased, concurrent with the down-regulation of miR-302b and Oct4. Our results suggest that miR-302b plays an important role in maintaining the pluripotency of ECCs and probably ESCs, by post-transcriptional regulation of Cyclin D2 expression.

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microRNAs (miRNAs) are small, endogenous RNAs of about 22 nucleotides that negatively regulate gene expression, mainly by translational repression [1,2], miRNAs play important roles in regulation of cell proliferation, differentiation, and maintenance of “stemness” [3–8], and play a particularly prominent role in development. Distinct sets of miRNAs, miR-302 cluster and miR-371 cluster, are specifically expressed in pluripotent human embryonic stem cells (hESCs) but not in differentiated embryoid bodies or adult tissues [9]. Very recently, it was found that the promoter of the miR-302 cluster was occupied by key ES cell transcriptional factors (Oct4, Sox2, and Nanog) in mouse and human cells [10–12] and that expression of the miR-302 cluster genes was down-regulated in response to Oct4 depletion [12]. The miR-302 family is also able to reprogram human skin cancer cells to a pluripotent ES cell-like state [13]. These data suggest that the gene cluster can be regulated by core transcription factors of ES cells and that it may play an integral role in the regulatory circuitry controlling ES cell “stemness”.

The family of D-type cyclins exhibits segmentally restricted expression pattern in hindbrain, and is, therefore, implicated in developmental regulation [14]. The D-type cyclins (Cyclin D1, D2, and D3) are cell cycle regulatory proteins that control specific cyclin-dependent kinases (CDKs) regulating the mid-G1 cell cycle checkpoint [15]. Gastrulation in rodents is associated with an increase in the rate of growth and with the initiation of differentiation within the embryo proper. Cyclin D1 is a target of miR-302a in hESCs (H1) [11], suggesting that the miR-302 cluster provides a link between key transcriptional factors and cell cycle regulation in ES cells.

Retinoic acid (RA) plays a critical role during normal development and regulates the decision between continued growth or differentiation in a variety of tumor cell lines [16]. In particular, strong evidence supports a role of RA in neuronal development.
[16,17], RA treatment of human embryonal carcinoma (hECC) NT2/D1 cells results in growth inhibition and neuronal differentiation, which can be monitored by the expression of markers such as neuronal specific cytoskeletal proteins and secretory or surface markers [18]. NT2/D1 cells specifically express the miR-302 gene cluster in a pattern similar to hESCs [9].

Here, we report that among the specific genes in the miR-302 cluster, mature miR-302b, derived from the antisense part of pre-miRNA, is highly expressed in undifferentiated NT2/D1 cells relative to RA-induced neuronal differentiated cells. In contrast, miR-302b* expressed from the sense part is barely detectable in undifferentiated cells. Expression of both mature miRNAs from either the sense or antisense strand of other pre-miRNA cluster genes (miR-302a*, miR-302a, miR-302c*, and miR-302c) is abundant in undifferentiated cells. We thus focused on effectiveness of miR-302b to mediate “stemness” or neuronal differentiation of NT2/D1 cells. Functional miR-302b is necessary to maintain self-renewal and inhibit neuronal differentiation of cells, by regulation of expression of Cyclin D2.

**Materials and methods**

**Cell culture, reagents, and transfection.** The NT2/D1 cell line was cultured in DMEM/F-12 medium, supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and glucose under humidified 5% CO2. For neuronal differentiation of NT2/D1 cells, 5 μM retinoic acid (RA) (Sigma) was added to the medium every day for 10 days. Anti-miR-302b (AM10081) and Pre-miR-302b (PM10081) (Ambion) were used for inhibition (AS-R-302b) and over-expression (miR-302b) of miR-302b. For all transfected cells, FITC-anti-miR-302b (*AS-R-302b) (Samchully Pharm. Co., Korea) or FAM-anti-miR negative control (*AS-R-Control) (AM17012) (Ambion) were used for FACS analysis, using a modular flow cytometer (Cytomation). For transfection of synthetic miRNAs,
using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s specification.

**Northern blot analysis and (quantitative) reverse transcription PCR ((Q)-RT-PCR) assays.** Total RNA samples were isolated from cultured cell lines as described previously [19,20]. Northern blots were performed as described [19,20] using Ultrahyb-Oligo hybridization buffer (Ambion) according to the manufacturer’s instruction. For the level of expression of miR-302b, QuantiMiR (SBI), and single tube TaqMan miRNA assays (Applied Biosystems) were used according to the manufacturer’s specification.

**microRNA microarray.** To identify the differentially expressed miRNAs in cells during differentiation, mirVana™ Array v.2 (Ambion) analysis was performed by Asuragen. We also utilized Genopal miRNA microarray system. Preparation of small-sized RNA, hybridization and signal detection were performed by Genopal protocol (Mitsubishi Rayon Co., Ltd., Japan) as previously described [21].

**Flow cytometric analysis.** For Tra-1-81 positive cell sorting, primary anti-Tra-1-81 mouse IgM antibody (Chemicon), and secondary APC-conjugated anti-mouse IgM (eBioscience) used and detected by flow cyt fluorimetry. For cell cycle assay, DNA of cells was stained with propidium iodide (50 μg/ml) and analyzed with FACS Calibur flow cytometer (BD Bioscience). Cell cycle analysis was performed using the ModFit program (Verity Software House, Inc.).

**Immunoblot and immunostaining assays.** For immunoblotting, antibodies were obtained from Santa Cruz Biotechnology [anti-Cyclin D1 (sc-246), anti-Cyclin D2 (sc-181), anti-p27 (sc-528)], R&D System (anti-Oct4), Covance (anti-Tuj1), and Sigma (anti-β-actin). Antibodies used for immunostaining were as follows: Santa Cruz biotechnology; anti-Oct4 (sc-5279), anti-Cyclin D2 (sc-181), and R&D Systems; anti-β-tubulin type III (TuJ1, BAM1195). The stained cells were visualized using secondary antibodies conjugated with FITC and Cy3 by confocal microscopy (Zeiss).

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Fig. 2. Effect of miR-302b on expression of stem cell marker genes in NT2/D1 cells. (A,B) Effect of miR-302b on mRNA (A) and protein (B) expression of stem cell marker genes and a neuronal gene (Pax6 and Tuj1) in cells using Q-RT-PCR (A) and Western analysis (B). “*” depicts FAM- or FITC-labeled AS RNAs. (C) The level of inhibition and over-expression of miR-302b by AS-R-Control and AS-R-302b using Q-RT-PCR. (D) Confocal images of immunofluorescence staining for Oct4 and Tuj1 expression (green) after transfection of miR-302b and AS-R-302b into cells. Nuclei were counterstained with 4’,6-diamidine-2’-phenylindole dihydrochloride (DAPI). Scale bar is 50 μm. (E) Analysis of expression levels of mature-miR-302b after AS-R-302b and miR-302b treatment by stem-loop Q-RT-PCR.
3′UTR luciferase reporter assays. Wild-type (WT) and mutant (Mut) UTR segments of miR-302b target were cloned into renilla/firefly dual-luciferase expression plasmid. One hundred nanomolar of miRNA was transfected together with 10 ng of luciferase reporter plasmid. There results were each transfected at least three times (on different days). Each transfected well was assayed in triplicate.

Results and discussion

In this study, NT2/D1 (hECC) cells are used as an in vitro model to investigate the molecular and biochemical regulation of ESCs by miRNAs, primarily because their miRNA expression patterns are similar to those of hESCs [9], our unpublished data. Cells express the stem cell marker Oct4 in the undifferentiated condition (no RA treatment; 0 day). Expression levels decreases during RA-induced neuronal differentiation and disappear 10 days after initiation of RA treatment (Fig. 1A). Known neuronal differentiation markers, NeuroD1 and Pax6 are expressed only after RA-induced neuronal differentiation (Fig. 1A) confirming the effectiveness of RA. Cells at 0 day and 10 days after RA treatment are used as undifferentiated (U) or differentiated (D) cells for further studies.

Undifferentiated NT2/D1-specific miRNAs

To identify the differentially expressed miRNAs in NT2/D1 cells during differentiation, the mirVana™ Array v.2 (Asuragen) microarray approach was performed on purified small RNAs (10–40 nt in size) from undifferentiated and 10-day-differentiated cells. The expression pattern of the differentially expressed miRNAs before and after differentiation was compared (Fig. 1B), indicating that the human miR-302 family, miR-422b and miR-187 are expressed at high levels in undifferentiated NT2/D1 cells relative to differentiated cells (blue boxes: “stemness”-specific miRNAs), whereas human miR-10b, miR-99a, miR-100, miR-181a&b, and miR-125b are up-regulated in differentiated cells relative to undifferentiated cells (red boxes: differentiation-specific miRNAs).

The “stemness”-specific miR-302 members (302b, 302c, 302a, and 302d) are all expressed at levels at least 16-fold higher in undifferentiated cells than in differentiated cells (Fig. 1C). miR-302c* and miR-302a* are also expressed more abundantly in undifferentiated cells relative to differentiated cells (between 8- and 16-fold difference) (Fig. 1C). However, miR-302b* is barely expressed in undifferentiated cells. We confirmed by Northern blot analysis that representative miRNA expression levels (Fig. 1D) are consistent with the array data (Fig. 1B). These results suggest that miR-302 antisense members (302b, 302c, 302a, and 302d) and some members of sense (302a* and 302c*) correlate with “stemness” character of undifferentiated cells, and that miR-302b shows the largest change in differential miRNA expression among family members during differentiation (Fig. 1C).

Expression of miR-302b is high in undifferentiated cells, and then abolished 10 days after initiation of RA treatment (Fig. 1E). The expression pattern of miR-302b is thus similar to that of Oct4 (Fig. 1A and E), suggesting the possibility that it may play a functional role in maintenance of “stemness”.

Necessity of miR-302b for maintenance of “stemness” in NT2/D1 cells

We evaluated the effects of inhibiting miR-302b function on expression of known stem cell marker genes using 2′-O-methyl antisense miR-302b RNA oligos (AS-R-302b). To obtain all cells containing AS-R-302b, we used AS-R-302b conjugated with FITC (*AS-R-302b) and then FACS sorted FITC-positive cells 2 days after transfection. FAM-labeled AS-R-Control (*AS-R-Control) was used as a negative control to characterize sequence-specific effects. *AS-R-302b-treated cells dramatically decrease expression of stem cell marker mRNAs: Oct4 (76 ± 4%, p-value <0.05), Nanog (75 ± 4%, p-value <0.05), and Sox2 (31 ± 5%, p-value <0.05) miRNAs (Fig. 2A). In addition this treatment increases expression of the differentiation marker Pax6 mRNA (86 ± 5%, p-value <0.05) (Fig. 2A). A decrease in expression of stem cell marker genes was not observed in cells treated with *AS-R-Control RNAs (scrambled sequence) relative to the untreated cells (data not shown) establishing the sequence specificity of the effect of miR-302b. In addition, miR-302b also affected the level of protein expression for stem cell marker genes. *AS-R-302b treated cells dramatically decrease expression of Oct4 protein (64 ± 3%) and increase expression of Tuj1 (40 ± 5%), a neu-
onal specific differentiation marker, compared to the control (*AS-R-Control) (Fig. 2 B).

We also examined the effect of over-expressing miR-302b using a synthetic miR-302b precursor (miR-302b) on “stemness” of cells. Over-expression of miR-302b resulted in a slight increase expression of Oct4 (~21 ± 4%) and Nanog (25 ± 5%) mRNAs in cells (Fig. 2A). These results imply that increased cellular miR-302b can result in expression of stem cell marker genes, and that expression of miR-302b in undifferentiated cells may already be near saturating levels and thus not to initiate a state of more “stemness”.

The effectiveness of miR-302b suppression by *AS-R-302b treatment of undifferentiated cells was confirmed by Q-RT-PCR analysis: 93 ± 3% suppression of miR-302b expression relative to a negative control (*AS-R-Control) (Fig. 2C). The over-expression of the mature form of miR-302b by precursor miR-302b (miR-302b) in cells was also confirmed by Q-RT-PCR relative to its negative control (miR-Control) (29 ± 5%) (Fig. 2C).

To further examine the effects of functional miR-302b on “stemness” of cells during RA-induced neuronal differentiation, we investigated the phenotype of cells after inhibition or over-expression of functional miR-302b by transfection of AS-R-302b or miR-302b oligonucleotide, respectively, and RA treatment for 3 days. Alteration of Oct4 and Tuj1 expression following treatment was examined by immunostaining (Fig. 2D). NT2/D1 cells reduced expression of Oct4 and differentiated into neural cells (Tuj1 expression, green) after RA treatment for 3 days (Mock, top panel). However, in the presence of miR-302b oligonucleotide, cells not only sustained the level of Oct4 expression, but also did not differentiate into neural cells (no Tuj1 expression) even after RA treatment for 3 days (Fig. 2D, middle panel). In contrast, when cells were transfected with AS-R-302b and induced to neuronal differentiation with RA, inhibition of functional miR-302b by AS-R-302 treatment resulted in reduction of Oct4 expression and induction of Tuj1 expression (Fig. 2D, bottom panel). Inhibition or enhancement of miR-302b expression by transfection of AS-R-302b or miR-302b oligonucleotides at 0 and 3 days after differentiation of cells was confirmed using Q-RT-PCR (Fig. 2E).

In summary, these data suggest that miR-302b is required for “stemness” in undifferentiated cells and is necessary for maintain-
ing expression of stem cell marker genes (Oct4, Nanog, and Sox2) as well as blocking expression of neuronal genes (Pax6 and TuJ1) in both transcriptional and translational levels.

Potential mechanism for maintenance of stemness of cells by miR-302b

To understand how miR-302b could play a direct role in regulation of “stemness” in undifferentiated cells and gain additional insight into its potential role in maintenance of expression for stem cell marker genes, we identified potential targets for miR-302b using a bioinformatics approach (miRanda: http://www.microrna.org/microrna/home.do). No stem cell marker genes were identified as potential targets of miR-302b, but many cell cycle-related genes were including Cyclin D1, Cyclin D2, RB1, and E2F2 (Supplement 1).

To examine the distribution of cell cycle phases during differentiation of cells, an analysis was performed on asynchronously proliferating cells at 0, 3, and 7 days after RA treatment (Fig. 3A). Undifferentiated proliferating cells typically reside ~48% in the S-phase compartment, but after RA treatment, growth of cells is arrested as evidenced by a decrease in S-phase cells to 38% (RA day 3) and a further decrease to 24% (RA day 7) as more cells accumulate in G1 (Fig. 3A).

Since D-type cyclins (Cyclin D1, D2, and D3) in conjunction with cyclin-dependent kinases CDK4 and CDK6 are known to be key regulators of growth control and the G1/S transition [15], we examined the expression pattern of Cyclin D1, D2, and D3 during differentiation of cells by RT-PCR and Western blotting (Fig. 3B). During differentiation, expression of Cyclin D1 is down-regulated, whereas expression of Cyclin D2 and Cyclin D3 is up-regulated (Fig. 3B). miRNAs negatively regulate gene expression at the post-transcriptional level mainly through translational repression by binding to the 3′UTRs of target genes even though in some cases, mRNA decay is initiated by miRNA-guided rapid deadenylation [22–24]. Since a potential target of miR-302b should be up-regulated at the post-transcriptional level during differentiation, expression of Cyclin D2 protein exhibits a pattern opposite to that of Oct4 (Fig. 3B) and miR-302b (Fig. 1F) suggesting that it may be a more likely miR-302b target. In addition, Cyclin D2 mRNA levels do not change (Fig. 3B, left panel). These data indicate that there is a strong possibility that Cyclin D2 is a target of miR-302b post-transcriptional regulation and this possibility would be consistent with the reciprocal down-regulation of Cyclin D2 protein.

To determine if miR-302b can directly bind to the 3′UTR of Cyclin D2 transcript and regulate its expression, we transfected cells with a dual (renilla and firefly) luciferase reporter construct (psiCHECK2, Promega) containing a perfect match for the miR-302b binding site (wild-type; WT) in the 3′UTR of cyclin D2 (Fig. 4A). The WT Cyclin D2 reporter showed an approximately three-fold inhibition of Renilla luciferase activity relative to a mutant reporter (mutant; Mut) with mismatched sequences inserted into the “seed” sequences of the predicted miR-302b-binding site (Fig. 4B).

However, co-transfection of the WT construct with AS-R-302 cells did not show any inhibition of luciferase activity. In addition, inhibition of functional miR-302b by transfection with AS-R-302b resulted in an increase of Cyclin D2 protein levels (Fig. 4C and Supplement 2), but no alteration of Cyclin D2 mRNA expression (Fig. 4C). These data indicate that the endogenous miR-302b in NT2/D1 cells directly binds to exogenous WT reporter gene and inhibits expression of the reporter and indicates that the co-transfection of AS-R-302b functionally abolishes miR-302b action.

To further investigate the relationship of Cyclin D2 expression with Oct4 and TuJ1 during differentiation we carried out immunostaining. Undifferentiated cells are positive for Oct4 immunostaining and negative for Cyclin D2 and TuJ1. In contrast, during differentiation, we see a reduction of Oct4 staining coincident with the appearance of Cyclin D2 and TuJ1 staining (Fig. 4D). This pattern is similar to the inhibition of functional miR-302b by transfection with AS-R-302b (Fig. 3C, Fig. 2B and D, and Supplement 2).

In summary, our results suggest that miR-302b plays an important role in maintaining the “stemness” hESCs (NT2/D1) and likely serves a similar purpose in hESCs: post-transcriptional regulation of Cyclin D2 expression.

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Appendix A. Supplementary data


References


