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

Nan Sook Lee [a,1], Jong Soo Kim [b,1], Wha Ja Cho [b], Man Ryul Lee [b], Riley Steiner [c], Andrea Gompers [d], Daijun Ling [e], Jae Zhang [e], Pl Strom [f], Mark Behlke [g], Sung-Hwan Moon [h], Paul M. Salvaterra [e], Richard Jove [a], Kye-Seong Kim [b,*]

[a] Division of Molecular Medicine, Beckman research Institute of the City of Hope, Duarte, CA, USA
[b] Department of Anatomy and Cell Biology and Department of Biomedical Science, College of Medicine, Hanyang University, Seoul, Republic of Korea
[c] Department of Biology and Department of Diplomacy and World Affairs, Occidental College, CA, USA
[d] Department of Biological Sciences, Califonia State University, Los Angeles, CA, USA
[e] Division of Neuroscience, Beckman research Institute of the City of Hope, Duarte, CA, USA
[f] Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway
[g] Integrated DNA Technologies, Inc., Coralville, IA, USA
[h] CHA Stem Cell Institute & CHA Biotech, Pochon CHA University, Seoul, Republic of Korea

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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.

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 pluropotent 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

* Corresponding author. Fax: +82 2 2281 7841.
E-mail address: ks66kim@hanyang.ac.kr (K.-S. Kim).
[1] These authors contributed equally to this work.

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[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 secretary 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 glutamine under humidified 5% CO_2. 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,

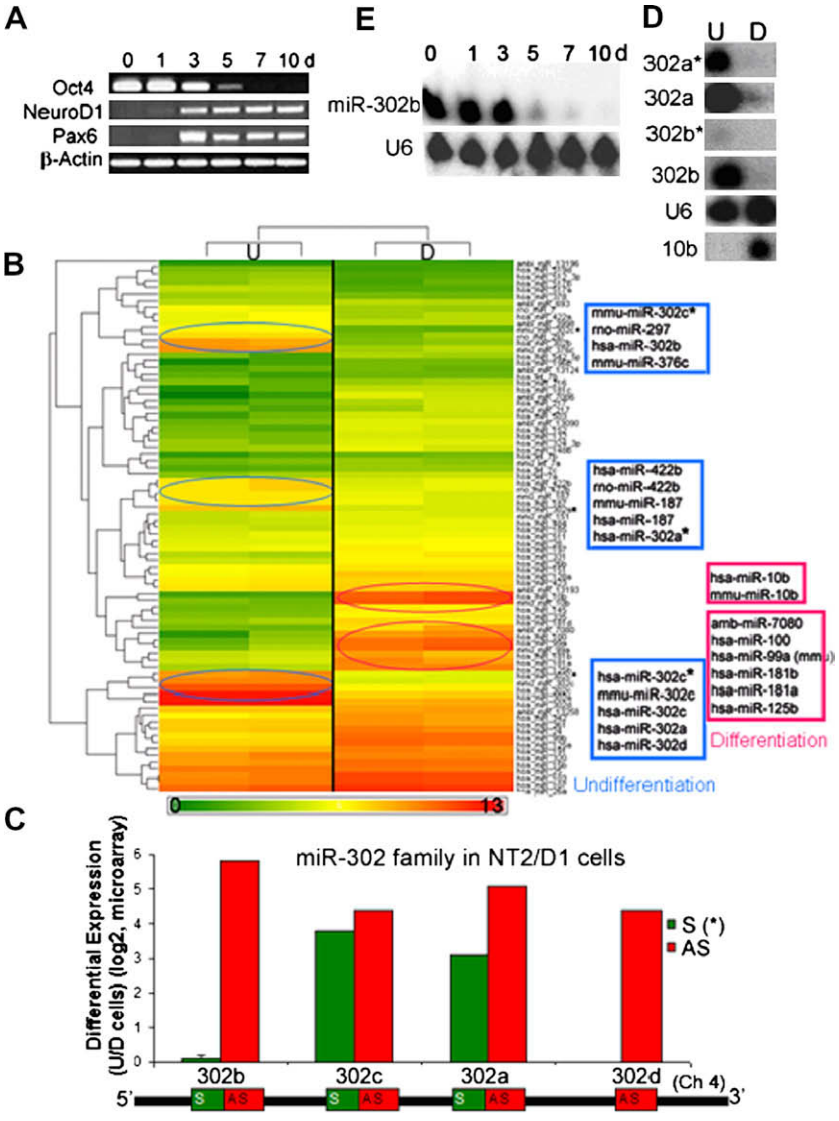

Fig. 1. Differential expression of miR-302 family in undifferentiated cells relative to differentiated ones. (A) Expression pattern of neuronal genes by RT-PCR in NT2/D1 cells after exposure to 5 μM retinoic acid (RA) for indicated number of days. Oct4 and β-actin are used as an undifferentiated (U) marker and a loading control, respectively. (B) miRNAs over-expressed in undifferentiated (U) and differentiated (D) cells using mirVana array. (C) Differential expression of miR-302 family in undifferentiated cells relative to differentiated ones. All antisense (a-d) and some sense (a*, c*) miR-302 family expressed mainly in undifferentiated cells and disappeared in 10 days' differentiated cells. (D) Expression of miRNAs in cells before (U) and after differentiation (D) using Northern blot analysis. Total RNAs were used from cells at 0 day (U) and 10 days (D) after RA treatment. (E) Expression of miR-302b in cells after exposure to RA for indicated days by Northern analysis. U6 is used as an internal control.