Stem Cell Research (2013) 11, 1299–1313

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MicroRNA-137 represses Klf4 and Tbx3 during differentiation of mouse embryonic stem cells

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Received 19 December 2012; received in revised form 5 September 2013; accepted 5 September 2013

Abstract MicroRNA-137 (miR-137) has been shown to play an important role in the differentiation of neural stem cells. Embryonic stem (ES) cells have the potential to differentiate into different cell types including neurons; however, the contribution of miR-137 in the maintenance and differentiation of ES cells remains unknown. Here, we show that miR-137 is mainly expressed in ES cells at the mitotic phase of the cell cycle and highly upregulated during differentiation. We identify that ES cell transcription factors, Klf4 and Tbx3, are downstream targets of miR-137, and we show that endogenous miR-137 represses the 3′ untranslated regions of Klf4 and Tbx3. Transfection of ES cells with mature miR-137 RNA duplexes led to a significant reduction in cell proliferation and the expression of Klf4, Tbx3, and other self-renewal genes. Furthermore, we demonstrate that increased miR-137 expression accelerates differentiation of ES cells *in vitro*. Loss of miR-137 during ES cell differentiation significantly impeded neuronal gene expression and morphogenesis. Taken together, our results suggest that miR-137 regulates ES cell proliferation and differentiation by repressing the expression of downstream targets, including Klf4 and Tbx3.

Introduction

Embryonic stem (ES) cells have the remarkable ability to replicate indefinitely while retaining the capacity to differentiate into functionally distinct cell types including neurons. The pluripotency and self-renewal of ES cells are regulated by a core transcriptional circuitry comprising of transcription factors, chromatin modulators, and small non-coding microRNAs (miRNAs) (Dejosez and Zwaka, 2012; Li et al., 2012; Pauli et al., 2011). The self-regulatory networks formed by transcription factors such as Oct4, Sox2, Nanog, Klf4, Esrrb, Tbx3, and Tcf3 regulate a wide range of downstream genes required for self-renewal and pluripotency (Boyer et al., 2005; Chambers and Tomlinson, 2009; Ivanova et al., 2006; Kim et al., 2008; Silva and Smith, 2008). In addition to the transcription factors, epigenetic changes associated with posttranslational modifications of histone proteins are known to regulate gene expression in ES cells (Li et al., 2012).

Abbreviations: ES cells, embryonic stem cells; NC, neuronally differentiated ES cells; EB, embryoid body; LIF, leukemia inhibitory factor; miRNA, microRNA; 3′UTR, 3′ untranslated region; LSD1, lysine specific demethylase 1; Tuj1, neuronal class III β-tubulin 3; RT, reverse transcription; qPCR, quantitative real-time polymerase chain reaction; ddPCR, droplet digital PCR; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; PBS, phosphate buffered saline; SSC, saline-sodium citrate.

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http://dx.doi.org/10.1016/j.scr.2013.09.001

Moreover, by directly interacting with ES cell transcription factors, miRNAs are shown to regulate the central signaling circuitry in ES cells (Pauli et al., 2011). The genetic deletion of key miRNA processing enzymes such as Dicer or Dgcr8 has been shown to impair cell cycle progression and cause defective differentiation (Murchison et al., 2005; Wang et al., 2008). Studies on the role of miRNAs in ES cells suggest that the regulatory function of the miRNA is often regulated through, and controlled by, various transcription factors important for self-renewal and pluripotency (Marson et al., 2008; Melton et al., 2010; Tay et al., 2008; Xu et al., 2009). An understanding of the interactions among these factors will be critical for the development of improved strategies to reprogram differentiated cells or induce differentiation of pluripotent ES cells to functionally distinct cell types.

miRNAs are short non-coding RNAs that can modulate gene expression by binding to the complementary sequences on target sites in the 3′ untranslated regions (UTRs) of messenger RNA (Bartel, 2004). There is strong evidence that miRNAs play an important role in the maintenance of ES cells (Pauli et al., 2011). In ES cells, a majority of the miRNA species are produced by 4 miRNA loci (the miR-21, the miR-17-92 cluster, the miR-15b-16 cluster and the miR-290-295 cluster) that regulate cell cycle progression and oncogenesis (Calabrese et al., 2007). During differentiation, a number of miRNAs exhibit distinct expression patterns and have been shown to fine tune or restrict cellular identities by targeting important transcription factors or key pathways (Stefani and Slack, 2008). For example, miRNA-145 has been shown to repress Oct4, Sox2, and Klf4 during differentiation of human ES cells into mesoderm and ectoderm lineages (Xu et al., 2009). Furthermore, miR-134, miR-296, and miR-470 target the transcription factors Nanog, Oct4, and Sox2 to promote retinoic-acid-induced differentiation of mouse ES cells (Tay et al., 2008). The contribution of additional miRNAs involved in the direct repression of ES cell transcription factors during neuronal differentiation remains unknown. We searched for miRNAs that show increased expression levels during neuronal differentiation and are predicted to target ES cell genes. The expression of miR-137 has been found to increase during differentiation of neural stem cells into neurons (Sun et al., 2011b; Szulwach et al., 2010). Moreover, miR-137 is one of the two miRNAs in ES cells that are co-occupied by the key transcription factors: Oct4, Sox2, and Nanog (Boyer et al., 2005). These findings suggest that miR-137 may be an important component of the transcriptional regulatory circuitry, and that temporal expression of miR-137 may influence proliferation and differentiation of ES cells.

Here, we show that miR-137 is mainly expressed in ES cells at mitotic stages of the cell cycle and is significantly upregulated when ES cells are differentiated into neuronal lineage. We demonstrated that miR-137 directly targets the ES cell transcription factors, Klf4 and Tbx3, as well as a known miR-137 target, lysine specific demethylase 1 (LSD1) (Balaguer et al., 2010; Sun et al., 2011b). The repressive effect of miR-137 is mediated by its binding to the target sites present in the 3′ untranslated regions (3′UTRs) of the mRNAs. Furthermore, we found that miR-137 disrupts ES cell self-renewal and accelerates differentiation of ES cells *in vitro*.

Materials and methods

ES cell culture and differentiation

R1 ES (ATCC) and Dgcr8$^{-/-}$ (Novus Biologicals) cells were maintained on mitomycin C treated MEF feeder layers and differentiated *in vitro* as described previously (Melton et al., 2010; Nair, 2006). Briefly, undifferentiated ES cells were grown on gelatin-coated tissue culture plates in the presence of 1000 U/ml leukemia inhibitory factor (LIF; Chemicon) in ES cell medium. To induce embryoid body (EB) formation, the cells were trypsinized and dissociated to a single-cell suspension and plated in ES cell medium without LIF onto non-adherent bacterial culture dishes at a density of 2–2.5 × 10^6 cells/dish. Four-day-old EBs were plated onto adherent tissue culture dishes and maintained in ES cell medium for 24 h. The selection of nestin-positive cells was initiated by replacing the ES cell medium with serum-free insulin/transferrin/selenium medium. Cells grown in differentiation medium for 12–14 days were used in all experiments.

Western immunoblotting

Total cell extracts were prepared and Western immunoblotting was carried out as described in our previous publication (Nair et al., 2012). Western blots of Klf4, Tbx3, LSD1, CoREST, RXRα, GAPDH, and β-actin were performed using anti-Klf4 (Millipore; 09-821), anti-Tbx3 (SCB: sc-48781), anti-LSD1 (CST; 2139), anti-CoREST (Millipore; 07-455), anti-RXRα (SCB; sc-553), anti-GAPDH (SCB; sc-166545), and β-actin (SCB; sc-166545) antibodies. All horseradish peroxide-conjugated secondary antibodies were from Santa Cruz Biotechnology.

Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) was carried out to measure gene expression as described in our previous publication (Nair et al., 2012). Briefly, RNA was isolated using an Absolutely RNA preparation kit (Agilent Technologies) and reverse transcribed using the Superscript™ III first-strand synthesis system (Invitrogen). SYBR green qPCR reactions on resulting cDNAs were performed on an ABI 7900HT (Applied Biosystems). Gene-specific primers used for RT-qPCR are shown in Table 1. β-Actin, α-tubulin, and Rps11 were used as internal normalization controls.

For RT-qPCR assays of miRNAs, total RNA was prepared using mirVANA miRNA isolation kit (Ambion). cDNA was synthesized from 100 ng of total RNA using miRNA specific RT primers and the expression was quantified using TaqMan MicroRNA Kits according to the manufacturer's protocol (Applied Biosystems). RT-qPCR assays using TaqMan Assay Kits, mmu-miR137 (ID 001129), hsa-miR16 (000396), and mouse snoRNA (001232) were used to determine the expression of miR-137, miR-16, and snoRNA, respectively. Relative fold mRNA and miRNA levels were determined by the $2^{-\Delta\Delta CT}$ (cycle threshold) method (Nair et al., 2012). The expression levels of each miRNA in undifferentiated ES cells (ESC) were arbitrarily set to one.