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Mature Let-7 miRNAs fine tune expression of LIN28B in pluripotent human embryonic stem cells

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ABSTRACT

MicroRNAs (miRNA) are central regulators of diverse biological processes and are important in the regulation of stem cell self-renewal. One of the widely studied miRNA-protein regulators is the Lin28-Let-7 pair. In this study, we demonstrate that contrary to the well-established models of mouse ES cells (mESC) and transformed human cancer cells, the pluripotent state of human ES cells (hESC) involves expression of mature Let-7 family miRNAs with concurrent expression of all LIN28 proteins. We show that mature Let-7 miRNAs are regulated during hESC differentiation and have opposite expression profile with LIN28B. Moreover, mature Let-7 miRNAs fine tune the expression levels of LIN28B protein in pluripotent hESCs, whereas silencing of LIN28 proteins have no effect on mature Let-7 levels. These results bring novel information to the highly complex network of human pluripotency and suggest that maintenance of hESC pluripotency differs greatly from the mESCs in regard to LIN28-Let-7 regulation.

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1. Introduction

The stem cells extracted from the inner cell mass of an embryo are pluripotent, i.e. they have unique ability of long-term self-renewal and the potential to develop into all specialized cell types (Evans and Kaufman, 1981; Martin, 1981). However, embryonic stem cells with different origins have different characteristics. Mouse and human embryonic stem cells (mESCs and hESCs, respectively) are distinguished for example, by their morphology, marker gene expression, and culture requirements (Xue et al., 2011). Also, different pluripotent states of embryonic cells have been identified in both species at different stages of pre- and postimplantation embryos. Epiblast stem cells (EpiSC) (Brons et al., 2007) from mouse postimplantation embryo are considered pluripotent, however, but exhibit limited differentiation potential and the characteristics more resemble hESCs than mESCs (Nichols and Smith, 2009; Tesar et al., 2007). It has been proposed that mESCs represent the naïve, ground state pluripotency, whereas EpiSCs and hESCs are primed pluripotent cells. Recent studies have also succeeded in establishing naïve human stem cell cultures that differ from hESCs and have mESC characteristics (Chan et al., 2013; Gafni et al., 2013; Hanna et al., 2010; Takashima et al., 2014; Theunissen et al., 2014; Ware et

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al., 2014), but more closely resemble human preimplantation blastocyst (Huang et al., 2014).

Micro RNAs (miRNA) are small non-coding RNA molecules acting primarily in translational repression. Interestingly, different pluripotent states can be discriminated by their miRNA profiles (Jouneau et al., 2012; Neveu et al., 2010). The family of Let-7 miRNAs is highly expressed in somatic cells, and repression of Let-7 is thought to be important in establishing the pluripotent state (Melton et al., 2010; Viswanathan and Daley, 2010). Let-7 together with Lin28 has been reported to form a bistable switch in mice and nematodes. This double negative feedback loop is thought to stabilize and determine different cellular fates, Lin28 in establishing the undifferentiated and Let-7 the differentiated cell state (Shyh-Chang and Daley, 2013; Viswanathan and Daley, 2010). Human LIN28 protein has two paralogs, LIN28A (also LIN28) and LIN28B, and both have been shown to have a role in pluripotency and cell reprogramming (Qiu et al., 2010; Yu et al., 2007; Zhang et al., 2016). LIN28 proteins have also been shown to be important in cancers and to inhibit Let-7 biogenesis. However, there is increasing evidence that LIN28 proteins have also Let-7 independent functions (Mayr and Heinemann, 2013; Shyh-Chang and Daley, 2013).

Here, we provide evidence that challenges the LIN28-Let-7 double negative feedback-loop mechanism in human ES cells. We show that mature Let-7 miRNAs are present and have a function in pluripotent hESCs in finetuning the expression of LIN28B, and that silencing LIN28 proteins has no effect on mature Let-7 miRNAs.

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2. Materials and methods

2.1. Cell culture and differentiation series

H7, H9 (WiCell Research Institute, Madison, WI) and HS360 (Outi Hovatta, Karolinska Institutet, Sweden) were used for experiments in feeder free conditions on Matrigel (BD Biosciences) in mTeSR1 (Stem Cell Technologies). Cells were passaged by using type IV collagenase (Gibco) or Dispase (Stem Cell Technologies). Embryonal carcinoma cell line NT2D1 (from Peter W. Andrews, University of Sheffield, UK) was grown in DMEM (Sigma) supplemented with 10% fetal calf serum (FCS) (PromoCell) and 2 mM L-glutamine (Sigma). Spontaneous EB differentiation series were made by scraping the cells from Matrigel plates and transferring to non-coated cell culture plates. The cells were grown in normal ES culture medium for 2–3 days, after which ES medium without bFGF was added. Medium was changed every 2–3 days.

2.2. Flow cytometry and cell sorting

Pluripotent cell population was sorted using antibody against SSEA3 cell surface marker, details of the antibodies are given in Supplemental information. Cells were harvested with trypsin. Sorting was performed with BD FACSAria Ilu cell sorter (BD) and FACSDiva Software.

2.3. RT-qPCR analysis

Total RNA was extracted using TRI Reagent (Molecular Research Center Inc) and chloroform (Sigma-Aldrich). Mature miRNAs were analyzed with Taqman MicroRNA Assays (listed in Supplemental information), Tagman MicroRNA Reverse Transcription Kit (PA4366597) and Tagman universal master mix No AmpErase UNG (PN 4324018) according to manufacturer's protocols (all from Applied Biosystems). For mRNA analysis 1 µg of total RNA was DNase treated twice [DNase I Amplification Grade (Invitrogen)] and analyzed with reference gene EF1 α to confirm no DNA was left in the sample. For cDNA synthesis Superscript II (Invitrogen) was used. The primer and probe sequences were designed using Universal ProbeLibrary Assay Design Center and are listed in Supplemental information. MicroRNA and mRNA gene expression levels were measured using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Expression levels were normalized using housekeeping genes. Change in threshold cycles (Ct) for each gene was calculated $\Delta Ct = Ct(gene) - Ct(housekeeping gene)$. Relative expression to control sample was calculated as fold change FC = $(2^{-\Delta Ct})$.

2.4. Protein analyses

Protein analysis was carried out as previously described (Narva et al., 2011). The details of the antibodies are given in Supplemental information.

2.5. Transfections, silencing and gain of function studies

Transfections were performed according to the manufacturer's protocols using Lipofectamine RNAiMax reagent (Invitrogen). Silencing of miRNAs was performed with miRCURY LNA™ microRNA Inhibitors (Exiqon). For gain of function studies Pre-miR miRNA Precursors (Ambion, Life Technologies) were used according to manufacturer's protocol. Oligonucleotides used in this study are listed in Supplemental information.

2.6. Sequencing data analysis

Sequencing of samples was carried out at Fasteris (Switzerland). Sequences were mapped to the hg19 genome reference using bowtie allowing for a maximum of two mismatches in the seed region (length of 17 nt) and discarding reads that map to more than five genomic locations. The alignments were then used as input to miRDeep2 to generate the coverage for mature microRNAs. Default parameter values were used. In cases where a mature microRNA corresponded with multiple precursor microRNAs, only the counts from the precursor microRNA with the highest number of mapped reads were included. Reads per million microRNAs mapped (rpm) were obtained and averaged for the three replicates. GEO accession number GSE55757.

3. Results and discussion

3.1. Mature Let-7 miRNAs are expressed in the pluripotent hESCs

Contrary to the studies of mESCs, we detected expression of all mature Let-7 miRNA family members in pluripotent hESCs in our genome wide deep sequencing data (Supplementary material Table 1). The pluripotency and differentiation associated miRNAs showed the expected expression profiles in the dataset (Morin et al., 2008; Suh et al., 2004). We further validated the sequencing results in two different hESC lines (HS360, H9) by using RT-qPCR designed to specifically detect the mature miRNAs. Somatic fibroblasts were used as a positive control for Let-7 expression, and NT2D1 embryonal carcinoma cells (EC), malignant counterparts of hESCs, as positive control for the miRNAs reported to be expressed in pluripotent cells (Suh et al., 2004) (miR-302-367, miR-21) (Fig. 1A). The RT-qPCR results confirmed Let-7a, -b, -g and pluripotency miRNAs were expressed in hESCs. The expressions of differentiation induced miRNAs, miR-122 and miR-199a (Morin et al., 2008), were low or undetectable as expected (Fig. 1A). Recent deep sequencing and microarray studies in fact supports our findings on mature Let-7 expression in pluripotent hESCs (Arefian et al., 2011; Lakshmipathy et al., 2007; Letzen et al., 2010; Morin et al., 2008; Ren et al., 2009; Stadler et al., 2010). Interestingly, we detected Let-7s to be simultaneously expressed with hESC specific cell cycle regulating (ESCC) miRNAs; miR-302b and -367 (Card et al., 2008; Lipchina et al., 2011) whereas in mouse ES cells, ESCC orthologues and Let-7 miRNAs are never co-expressed at high levels (Melton et al., 2010).

Pluripotent hES cells are relatively laborious to maintain in culture and typically a proportion of spontaneously differentiating cells exist in the cell population. As expression of mature Let-7 miRNAs has been reported to increase during cell differentiation (Patterson et al., 2012; Viswanathan et al., 2008) we wanted to exclude the possibility that the expression of mature Let-7 is coming from the spontaneously differentiated cells existing in the cell population. Thereby, we sorted the hESCs into pluripotent SSEA3 + (Draper et al., 2004) and spontaneously differentiated SSEA3 – cell populations (Fig. 1B). RT-qPCR analysis of sorted cells revealed expression of mature Let-7 miRNAs in both SSEA3 + and SSEA3 - cells (Fig. 1C). Importantly, mature Let-7 miRNAs were co-expressed in SSEA3 + cells with both LIN28 proteins along with pluripotency markers OCT4 and L1TD1 (Narva et al., 2011) (Fig. 1D,E). This was unexpected, since the Lin28 proteins have been shown to inhibit the biogenesis of Let-7 miRNAs in mouse ES and EC cells and in transformed human cell lines (Newman et al., 2008; Piskounova et al., 2011; Viswanathan et al., 2008). In addition, the expressions of LIN28A and LIN28B have been suggested to be mutually exclusive (Piskounova et al., 2011) and the proteins are not co-expressed in several human cancer cell lines (Viswanathan et al., 2009). Taken together, our results demonstrate that mature Let-7 miRNAs are expressed and co-exist with both LIN28 proteins in the pluripotent hESCs.

It has been demonstrated that hESCs resemble more EpiSCs than mESCs (Brons et al., 2007; Tesar et al., 2007). Further, mESC and EpiSCs express different sets of miRNAs and these cell types can be distinguished based on their miRNA profiles. Notably, higher levels of mature Let-7, miR-302d and miR-367 expressions have been observed in EpiSC than in mESCs (Jouneau et al., 2012). We examined the Let-7 levels in the novel miRNA data on naïve and primed human ES cells (Sperber et al., 2015). Remarkably, all Let-7 family members were expressed both in naïve and primed human ES cells in the data of Sperber et al.



Fig. 1. Mature Let-7 miRNAs are expressed in pluripotent human embryonic stem cells. (a) Validation of the sequencing results using RT-qPCR. Representative averaged data of two biological replicates of each cell line. Mean \pm SD. Threshold cycle Ct, Δ Ct = Ct(gene) – Ct(housekeeping gene) ND: not detected. Fib: fibroblast cells. (b) Representative flow cytometric analysis of cell sorting based on SSEA3 surface marker. In the figure is the secondary antibody staining alone, staining of hESCs (HS360) with SSEA3 before and after sorting. (c) RT-qPCR analysis of selected miRNAs in the SSEA3 + and SSEA3 – populations. Averaged data is from three biological replicates (HS360). Mean \pm SD. (d) Representative protein expression analysis of the pluripotency factors in the sorted cell populations (e) RT-qPCR analysis of selected mRNAs in the SSEA3 – hESC populations. Averaged data is from two biological replicates (HS360). Mean \pm SD.

Interestingly, Let-7a expression is significantly higher in primed hESCs than in naïve hESCs, whereas all the other family members have an opposite expression pattern. This data suggests that the difference in Let-7 expression between mouse and human pluripotent cells might be due to species-specific differences rather than the difference in the developmental state of pluripotent cells.

3.2. Mature Let-7 miRNAs are regulated and have opposite expression profiles with LIN28B during hESC differentiation

After detecting the mature Let-7 miRNAs in pluripotent hESCs we studied their expression in response to cell differentiation. In spontaneously differentiated hESCs the expressions of pluripotency markers were significantly lower (Fig. 2A, B, C). Analysis of Let-7 levels from the same samples revealed the downregulation of Let-7a, -b, and -g, in the first stages of the embryonic body (EB) differentiation (until day 15), followed by an increase in the expression in later time points (Fig. 2D). All analyzed family members had similar expression pattern. Supporting our data, similar expression pattern of Let-7b and -i were observed also in a study from Ren et al. (Ren et al., 2009). Interestingly, during differentiation the expression of Let-7 miRNAs had the opposite profile of the LIN28B (Fig. 2A) that were first upregulated until day 15, followed by reduction after day 20 when the miRNA levels started to increase. Moreover, LIN28B isoforms 1 and 2 were regulated with different kinetics. The mRNA level analysis of the same samples revealed that the kinetics of LIN28B downregulation was slower than the reductions in OCT4 and LIN28A levels. LIN28B levels started to slowly decay after day 10 and reduced 57% by day 25, whereas OCT4 was significantly downregulated already on day 10 and was reduced 97% by day 25 (Fig. 2B). Importantly, LIN28B was not significantly induced at mRNA level although upregulation was detected at the protein levels with both isoforms until day 15 and with LIN28B1 until day 25 of differentiation. This indicates post-transcriptional regulation of the LIN28B protein expression, possibly by Let-7 miRNAs.

3.3. Let-7 miRNAs fine tune the expression levels of LIN28B in pluripotent hESCs

Next, we studied whether mature Let-7 miRNAs are functional in hESCs and performed a series of loss- and gain of function studies. We used LNA microRNA inhibitors for silencing (Let-7a independently, or targeting the Let-7 family with 3 oligonucleotides) and Pre-miR miRNA precursors (Let-7a) for induction. The effects were studied by analysing expression levels of known or predicted targets of Let-7 miRNAs.

Silencing of Let-7 resulted in a clear increase in LIN28B protein levels, while the effect on LIN28A was only moderate (Fig. 3A, B, C). The effect on LIN28B expression was detected with all LNA oligonucleotides targeting the whole family of Let-7 miRNAs. Consistent with these results in public databases, (http://www.targetscan.org/, http://www.microrna.org/) human LIN28B has four predicted target sites for Let-7 miRNAs in its 3'UTR, while LIN28A has only one. This suggests that in human cells, LIN28B is a stronger target for Let-7 than LIN28A. The protein levels of pluripotency factors OCT4 or L1TD1 were not consistently affected in response to Let-7 silencing. Also, the analysis of mRNA levels from the samples did not show significant changes in the expression levels (data not shown). Whereas silencing of Let-7 led to an increase in LIN28B protein levels, induction of precursor Let-7a into the cells led to a decrease in LIN28B protein expression as expected (Fig. 3F).

These results show Let-7 to have a function in hESCs in fine tuning the expression of at least LIN28B protein levels. Most likely Let-7 miRNAs have also several other targets in hESCs that remain to be identified. Our results also show evidence that LIN28 proteins respond differentially to the silencing and induction of Let-7 indicating independent functions and differential roles for LIN28A and LIN28B in hESCs. Moreover, these results suggest that the level of Let-7 in hESCs needs to be tightly regulated to sustain the balance between these factors.

3.4. Silencing of LIN28 proteins has no effect on mature Let-7 levels

Finally, we examined whether silencing of LIN28A or LIN28B, independently or together, has an effect on mature Let-7 levels in hESCs. The fact that LIN28 proteins and mature Let-7 miRNAs co-express in these cells, suggests Let-7 independent functions. Supporting our previous observations and contrary to published results in mES cells (Viswanathan et al., 2008; Viswanathan et al., 2009) we did not see any effect on expression of mature Let-7a and Let-7g miRNAs in response to LIN28 knockdowns (Fig. 3D, E). These results are in concordance with several recent studies that have suggested Let-7 independent functions for LIN28 proteins in embryonic stem cells (Cho et al., 2012; Peng et al., 2011; Shyh-Chang and Daley, 2013;



Fig. 2. Mature Let-7 miRNAs are regulated and have opposite expression profiles with LIN28B proteins during human embryonic stem cell differentiation. (a) Representative protein level analysis of the pluripotency factors in spontaneous embryoid body (EB) differentiation. LIN28B has two isoforms, longer LIN28B1 28 kDa and shorter LIN28B2 20 kDa. Numbers below represent the quantitated protein expression compared to day 0 time point and balanced with β -actin (b) RT-qPCR analysis of *OCT4*, *LIN28A*, and *LIN28B* mRNAs in response to differentiation. Averaged data from three replicate cultures (H9, HS360) Mean \pm SD. **p < 0.01, ***p < 0.001. (c, d) RT-qPCR analysis of the levels of indicated miRNAs in response to cell differentiation. Averaged data from two biological replicates (H9) Mean \pm SD.



Fig. 3. Let-7 miRNAs fine tune LIN28B levels in pluripotent human embryonic stem cells but silencing of LIN28 proteins does not have an effect on mature Let-7 levels. (a) Representative western blot analysis of LNA silencing of Let-7a and control (H7). Numbers represent the relative protein expression levels compared to the control sample and balanced with β -actin. Day 1 time point. (b) Representative western blot analysis of indicated proteins in response to silencing of Let-7 family with three different LNA oligonucleotides (H9). (c) Averaged quantitated differences in the protein level expression of indicated proteins in response to Let-7a silencing with LNA oligonucleotide from two biological replicates (H7, H9). (d) Representative protein level analysis of LIN28A, B or both (H9). Numbers below represent the quantitated protein expression compared to non-targeted (NT) siRNA sample balanced with KU70. (e) RT-qPCR analysis of Let-7g glevels in response to silencing of LN28A, B, or both in hESCs. Averaged data is from three biological replicates (H7, H9), differences (H7, H9), differences in the relative protein level analysis of indicated proteins in response to silencing of LN28A, B, or both in hESCs. Averaged data is from three biological replicates (H7, H9), they shall be acting the protein level analysis of indicated proteins in response to introduction of Precursor Let-7a into hESCs (H9) on day 2 time point. Numbers represent the relative protein expression compared to the control sample and balanced with β -actin.

Wilbert et al., 2012). The alternative role for LIN28 proteins has been shown to be in translational regulation of messenger RNAs (Hafner et al., 2013; Peng et al., 2011; Wilbert et al., 2012). Interestingly however, a recent study (Triboulet et al., 2015) reported a single Let-7 to bypass LIN28 mediated repression in human cancer cells. This observation brings further intricacy to LIN28-Let7 axis in development and disease, and underlines the complexity of these interactions and regulation. Moreover, even though our transient silencing with siRNA is efficient and we do not see an effect on mature Let-7 levels even after double knockdown of LIN28, there is still a possibility that the residual LIN28 protein is sufficient to regulate Let-7 biogenesis.

3.5. Conclusions

In summary, our data shows that the pluripotent state of hESCs involves concurrent expression of LIN28 proteins and Let-7 miRNAs. Further, Let-7 miRNAs have a function in hESCs in fine tuning LIN28B protein levels. We suggest that maintenance of hESC pluripotency differ greatly from the mESCs in regard to LIN28-Let-7 expression profiles and interactions and that hESCs lack the double negative feedback loop formed by LIN28-Let-7. How this LIN-Let-7 pair function in naïve hESCs or EpiSCs remains to be studied. Analysing all the stem cell types would be needed to shed light if the results reported here are due to a difference in the developmental state of the cells or due to species-specific difference. Nonetheless, these results bring novel aspects to the complex network of pluripotency.

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Author contributions

N.R. performed majority of the experiments with the assistance of S.E. and M.R.E. A.S. performed part of siRNA cultures and protein quantifications. N.R. designed and interpreted the experiments with input from S.E., E.N., M.R.E., and R.L. M.M. and H.L. performed the analysis of sequencing dataset. H.R-B provided early access to miRNA dataset on naïve and primed hESCs. N.R. wrote the manuscript with final approval from all authors. R.L., H.L guided the research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.scr.2016.09.025.

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