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Identification of MicroRNAs Regulating Reprogramming Factor *LIN28* in Embryonic Stem Cells and Cancer Cells*[S]

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LIN28* (a homologue of the *Caenorhabditis elegans lin-28* gene) is an evolutionarily conserved RNA-binding protein and a master regulator controlling the pluripotency of embryonic stem cells. Together with *OCT4*, *SOX2*, and *NANOG*, *LIN28* can reprogram somatic cells, producing induced pluripotent stem cells. Expression of *LIN28* is highly restricted to embryonic stem cells and developing tissues. In human tumors, *LIN28* is up-regulated and functions as an oncogene promoting malignant transformation and tumor progression. However, the mechanisms of transcriptional and post-transcriptional regulation of *LIN28* are still largely unknown. To examine microRNAs (miRNAs) that repress *LIN28* expression, a combined *in silico* prediction and miRNA library screening approach was used in the present study. Four miRNAs directly regulating *LIN28* (*let-7*, *mir-125*, *mir-9*, and *mir-30*) were initially identified by this approach and further validated by quantitative RT-PCR, Western blot analysis, and a *LIN28* 3′-UTR reporter assay. We found that expression levels of these four miRNAs were clustered together and inversely correlated with *LIN28* expression during embryonic stem cell differentiation. In addition, the expression of these miRNAs was remarkably lower in *LIN28*-positive tumor cells compared with *LIN28*-negative tumor cells. Importantly, we demonstrated that these miRNAs were able to regulate the expression and activity of *let-7*, mediated by *LIN28*. Taken together, our studies demonstrate that miRNAs *let-7*, *mir-125*, *mir-9*, and *mir-30* directly repress *LIN28* expression in embryonic stem and cancer cells. Global down-regulation of these miRNAs may be one of the mechanisms of *LIN28* reactivation in human cancers.

LIN28 is an evolutionarily conserved RNA-binding protein with two RNA-binding domains (a cold shock domain and retroviral type CCHC zinc finger motif), which was first characterized as a critical regulator of developmental timing in *Caenorhabditis elegans* (1, 2). The mammalian homologs of the *C. elegans lin-28* gene (*LIN28* and *LIN28B*) are important in processes such as embryogenesis (3), skeletal myogenesis (4), germ cell development (5, 6), and neurogliogenesis (7, 8). Genome-wide association studies have implicated the *LIN28B* locus in controlling both height and the timing of menarche in humans (9–13). This finding has been successfully phenocopied in a transgenic mouse model that expresses inducible *LIN28* (14). Increasing evidence suggests that *LIN28* may also be a master regulator controlling the pluripotency of ES cells (15–18). *LIN28*, together with *OCT4*, *SOX2*, and *NANOG* (the "reprogramming factors"), can reprogram somatic cells to induced pluripotent stem cells (19). Several reports have demonstrated that *LIN28* binds to mRNAs, regulating their stability and translation (4, 16, 17). In addition, *LIN28* can bind to the terminal loops of the precursor of the miRNA *let-7*, thereby blocking the processing of *let-7* into its mature form (7, 8, 20–26). Importantly, expression of *LIN28* is highly restricted to ES[2] cells as well as developing tissues, and its expression dramatically decreases as differentiation proceeds (2, 4, 5, 8, 14, 27–29). In human tumors, *LIN28/LIN28B* is up-regulated/reactivated and functions as an oncogene promoting malignant transformation and tumor progression (30–37). However, the transcriptional and post-transcriptional regulation of mammalian *LIN28* is still largely unknown.

miRNAs are endogenous ~18–25-nucleotide non-coding small RNAs that regulate gene expression in a sequence-specific manner via degradation of target mRNAs or inhibition of protein translation (38–40). With the exception of miRNAs within the Alu repeats, which are transcribed by RNA polymerase III (41), most miRNAs are derived from primary miRNA transcripts transcribed by polymerase II and containing a 5′ cap and a poly(A) tail (42, 43). The primary miRNA transcript is cleaved within the nucleus into a ~70-nucleotide hairpin precursor known as pre-miRNA by a multiprotein complex called Microprocessor, which is composed of the RNase III enzyme Drosha and the double-stranded RNA-binding domain protein DGCR8/Pasha (44–47). Next, the pre-miRNA is exported into the cytoplasm by Exportin-5 via a Ran-GTP-dependent mechanism (48–50). The pre-miRNA is further cleaved into the mature ~22-nucleotide miRNA-miRNA* duplex by an RNase III enzyme, Dicer, in association with its partners TRBP/Loquacious and PACT in human cells

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[2] The abbreviations used are: ES, embryonic stem; miRNA, microRNA; UTR, untranslated region; EB, embryoid body.

(51, 52). Subsequently, an RNA-induced silencing complex called RISC is assembled with Argonaute 2 (53, 54). The miRNA strand is selectively incorporated into RISC (55, 56) and guides the complex specifically to its mRNA targets through base-pairing interactions.

EXPERIMENTAL PROCEDURES

Cell Culture—The cancer cell lines A2780, T47D, MCF7, and HeLa were cultured in RPMI1640 (Cellgro) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Mouse embryonic fibroblasts were cultured in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen) with 10% FBS and 1% penicillin/streptomycin. The mouse embryonic stem cell lines R1 (ATCC) and C57BL/6J-693 (The Jackson Laboratory) were maintained on gelatin-coated flasks with mitomycin C-treated mouse embryonic fibroblasts in DMEM containing 15% ES cell FBS (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% MEM nonessential amino acids (Invitrogen), 1% penicillin/streptomycin, 0.1 mM 2-mercaptoethanol (Invitrogen), and 1000 units/ml mouse leukemia-inhibitory factor (Chemicon).

Plasmid Construction—A genome-wide miRNA expression library was generated in the laboratory of Dr. Qihong Huang (The Wistar Institute). The full-length sequence of the human *LIN28* 3′-untranslated region (3′-UTR) was cloned from human genomic DNA using the Expand High Fidelity PCR system (Roche Applied Science). The PCR product was ligated into the PCR2.1 TOPO cloning vector (Invitrogen) and then subcloned into the psiCHECK2 reporter vector (Promega). For mutagenesis of microRNA binding sites on reporter vectors, an overlap extension approach by PCR was used as described previously (57). Briefly, primers encompassing the site to be mutated were designed to have overlapping ends. The first round of PCR, yielding two fragments with mutated sequences at either end, was performed using the Expand High Fidelity PCR system with wild type reporter plasmid as template. The fragments were purified and annealed with each other for mutually primed extension. A full-length DNA fragment with the desired mutation was then produced via a second round of PCR. Purified DNA was digested and ligated with the reporter vector backbone.

Western Blot—Cells were lysed in mammalian protein extraction reagent (Pierce). After quantification using a BCA protein assay kit (Pierce), 15 μg of total protein was separated by 10% SDS-PAGE under denaturing conditions and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% nonfat milk (Bio-Rad) and then incubated with an anti-LIN28 primary antibody (1:10,000; Abcam), followed by incubation with an anti-rabbit secondary antibody conjugated with HRP (1:10,000; Amersham Biosciences) together with an HRP-conjugated primary antibody for β-actin (1:10,000; Sigma). Immunoreactive proteins were visualized using the LumiGLO chemiluminescent substrate (Cell Signaling).

Quantitative Real-time RT-PCR—Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed using a high capacity RNA-to-cDNA kit (Applied Biosystems) under conditions provided by the supplier. cDNA was quantified by RT-PCR on an ABI Prism 7900 sequence detection system (Applied Biosystems) using the primers listed in Table 1. PCR was performed using SYBR Green PCR core reagents (Applied Biosystems) according to the manufacturer's instructions. PCR amplification of the housekeeping gene GAPDH was performed for each sample as a control for sample loading and to allow normalization across samples.

TABLE 1
Primer sequences

Gene	Accession number	Primer sequence
hLIN28	NM_024674.4	Forward: caaaaggaaagagcatgcagaag Reverse: gcatgatgatctagacctccaca
mLin28	NM_145833.1	Forward: gttcggcttcctgtctatgacc Reverse: cttccatgtgcagcttgctct
mOct-4	NM_013633.2	Forward: atggcatactgtggacctca Reverse: agcagcttggcaaactgttc
mNanog	NM_028016.1	Forward: ctcatcaatgcctgcagtttttca Reverse: ctcctcagggcccttgtcagc
mGata6	NM_010258.3	Forward: acagcccacttctgtgttccc Reverse: gtgggttggtcacgtggtacag
mGata4	NM_008092.3	Forward: cctggaagacaccccaatctc Reverse: aggtagtgtcccgtcccatct
mBrachyury (T)	NM_009309.2	Forward: ctctaatgtcctcccttgttgcc Reverse: tgcagattgtctttggctactttg
mGSC	NM_010351.1	Forward: ttcgggaggagaaggtgga Reverse: cggcgaggcttttgagga
mFgf5	NM_010203.3	Forward: aaagtcaatggctcccacgaa Reverse: ggcacttgcatggagttttcc
mNestin	NM_016701.3	Forward: tgagggtcaggtggttctg Reverse: agagcagggagggacattc

TaqMan miRNA Profiling Fluidic Cards—Total RNA was extracted with TRIzol reagent (Invitrogen). 700 ng of total RNA was subjected to reverse transcription using the Megaplex RT primer pool (Applied Biosystems) and the Taqman microRNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. cDNA was loaded to the microfluidic card of Taqman miRNA panel A (Applied Biosystems), and quantitative PCR was performed following the manufacturer's instructions.

TaqMan miRNA Assay—Expression of mature miRNAs were analyzed using the TaqMan miRNA assay (Applied Biosystems) under conditions defined by the supplier. Briefly, single-stranded cDNA was synthesized from 5 ng of total RNA in a 15-μl reaction volume, using the TaqMan microRNA reverse transcription kit (Applied Biosystems). The reactions were incubated first at 16 °C for 30 min and then at 42 °C for 30 min and then inactivated by incubation at 85 °C for 5 min. Each cDNA generated was amplified by quantitative PCR using sequence-specific primers from the TaqMan microRNA assays on an Applied Biosystems 7900HT sequence detection system (Applied Biosystems). Each 20-μl PCR included 10 μl of 2× Universal PCR Master Mix (without AmpErase UNG), 1 μl of 20× TaqMan microRNA assay mix, and 2 μl of reverse transcription product. The reactions were incubated in a 384-well plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Cell Transfection—For transient transfections, cells were plated 24 h before transfection at 50% confluence. Plasmid and miRNA mimic transfections were performed with the FuGENE6 transfection reagent (Roche Applied Science) or Lipofectamine RNAiMAX (Invitrogen), respectively.

Lentiviral Transduction and Stable Cell Line Generation—Lentiviral vector and packaging vectors were transfected into the packaging cell line 293T (ATCC) using the FuGENE6 Transfection Reagent (Roche Applied Science). The medium was changed 8 h post-transfection, and the medium containing lentivirus was collected 48 h later. Tumor cells were infected with lentivirus in the presence of 8 μg/ml Polybrene (Sigma).

Reporter Assay—Cells were plated on a 24-well plate and transfected with 0.125 μg of reporter vector together with 0.25 μg of miRNA expression plasmid using the FuGENE6 transfection reagent. Alternatively, a sequential transfection method was used as follows. 30 nM miRNA mimics were transfected using Lipofectamine RNAiMAX; 24 h post-transfection, 0.125 μg of reporter vector was transfected using FuGENE6 transfection reagent. In both cases, 48 h after reporter vector transfection, cells were harvested, and reporter assays were performed using a dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. Reporter activity was measured on the Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific).

Immunohistochemistry and Confocal Image—Sections were sequentially incubated in 5% normal serum, rabbit anti-LIN28 antibody diluted at 1:500 overnight at 4 °C, and Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) at 1:150 for 1.5 h at room temperature. Sections were counterstained with DAPI (Vector). The image was collected using an Axiovert 200 M inverted microscope (Zeiss).

Embryoid Body (EB) Formation Assay—Undifferentiated ES cells were trypsinized and resuspended in ES cell culture medium without mouse leukemia-inhibitory factor. ES cell suspensions were applied to gelatin-coated dishes and incubated at 37 °C for 40 min to remove mouse embryonic fibroblast cells. ES cells were collected and resuspended in ES cell culture medium without mouse leukemia-inhibitory factor. For suspension cultures, 1×10^6 cells were placed in 100-mm Petri dishes, and the medium was changed every 2 days.

let-7-responsive Sensor Construction and Transfection—An miRNA-responsive sensor, a technique for monitoring miRNA activity, has been used to detect *let-7* expression in mouse embryos *in vivo* and in mammary epithelial progenitor cells *in vitro*. The sensor contains a constitutively expressed reporter bearing sequences complementary to *let-7* in the downstream region of the 3′-UTR of a reporter gene. In cultured cells transfected with the sensor, expression of the reporter is decreased when *let-7* is present. Thus, the *let-7*-responsive sensor is able to monitor *let-7* activity in real time. The *let-7* sensor was constructed by introducing two copies of *let-7b* perfect complement sequences into the 3′-UTR region of the *Renilla* luciferase gene in the psiCheck 2 vector (Promega). The *let-7*-complementary oligonucleotides (sense, TCG AGA ACC ACA CAA CCT ACT ACC TCA GGA TCC AAC CAC ACA ACC TAC TAC CTC AGC; antisense, GGC CGC TGA GGT AGT AGG TTG TGT GGT TGG ATC CTG AGG TAG TAG GTT GTG TGG TTC) were purchased from IDT and annealed in buffer containing 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, and 2 mM magnesium acetate. A2780 cells were seeded in 6-well plates and grown overnight to 40% confluence prior to transfection. To test *let-7* activity, a total of 1 μg of sense vector was introduced to the cells using FuGENE6 transfection reagent (Roche Applied Science). All transfection experiments were done in triplicate and repeated at least twice. Luciferase assays were performed 48 h post-transfection with a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific) using the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Statistics—Statistical analysis was performed using the SPSS statistics software package (SPSS, Chicago, IL). All results are expressed as mean ± S.D. and with significance at $p < 0.05$.

RESULTS

A Combined in Silico Prediction and miRNA Library Screening Approach Identified miRNAs Targeting LIN28—The expression of *LIN28* is highly restricted to ES cells, somatic progenitor cells, and developing tissues but is not detectable in most adult organs (2, 4, 5, 8, 14, 27–29). However, *LIN28* is dramatically up-regulated/reactivated in human cancers (30–35). There is a double negative regulation loop between *LIN28* and the miRNA *let-7* (7, 8, 20–26). To identify miRNAs regulating *LIN28*, our initial screening was performed on a platform containing four well characterized cancer cell lines, two of which (A2780 and T47D) highly expressed endogenous LIN28 and two of which (MCF7 and HeLa) were LIN28-negative (Fig. 1*A*). Consistent with previous reports (7, 8, 20–26), the levels of *let-7* in the LIN28-positive cell lines were remarkably lower than the levels in LIN28-negative lines (Fig. 1*B*).

Genome-wide miRNA library screening has been used successfully to identify miRNAs that regulate certain protein-coding genes (58, 59). Because this method is time- and labor-intensive, we designed a bioinformatics-driven screening approach, in which a concentrated miRNA library was generated by miRNA binding site prediction (*i.e.* we used bioinformatics for miRNA target prediction as a filter to generate a restricted/selected miRNA library on which to perform the functional screening). This allowed us to perform the experimental screening in a co-transfection system in which a luciferase reporter was co-transfected with a pre-miRNA-expressing vector (Fig. 1*C*). The reporter vector contained the full-length human *LIN28* 3′-UTR sequence, which was cloned downstream of the reporter gene *Renilla* (*hRluc*), such that the reporter gene expression was regulated by the *LIN28* 3′-UTR. The firefly luciferase (*hluc*) reporter served as an internal control for transfection efficiency (Fig. 1*C*). To test our screening system, we first chose the miRNA *let-7*, one of the known miRNAs regulating *LIN28*. As expected, *let-7* significantly reduced luciferase activity in LIN28-negative cell lines but not in LIN28-positive cell lines, in which the pre-miRNA maturation was blocked by endogenous *LIN28* (Fig. 1*D*).

Next, we predicted miRNA binding sites in the 3′-UTR of the human *LIN28* gene using the bioinformatics miRNA target prediction program TargetScan (available on the World Wide Web) (60). A total of 23 potential miRNA binding sites that were broadly conserved among vertebrates were identified (Fig. 1*E*). The individual miRNA expression vectors for

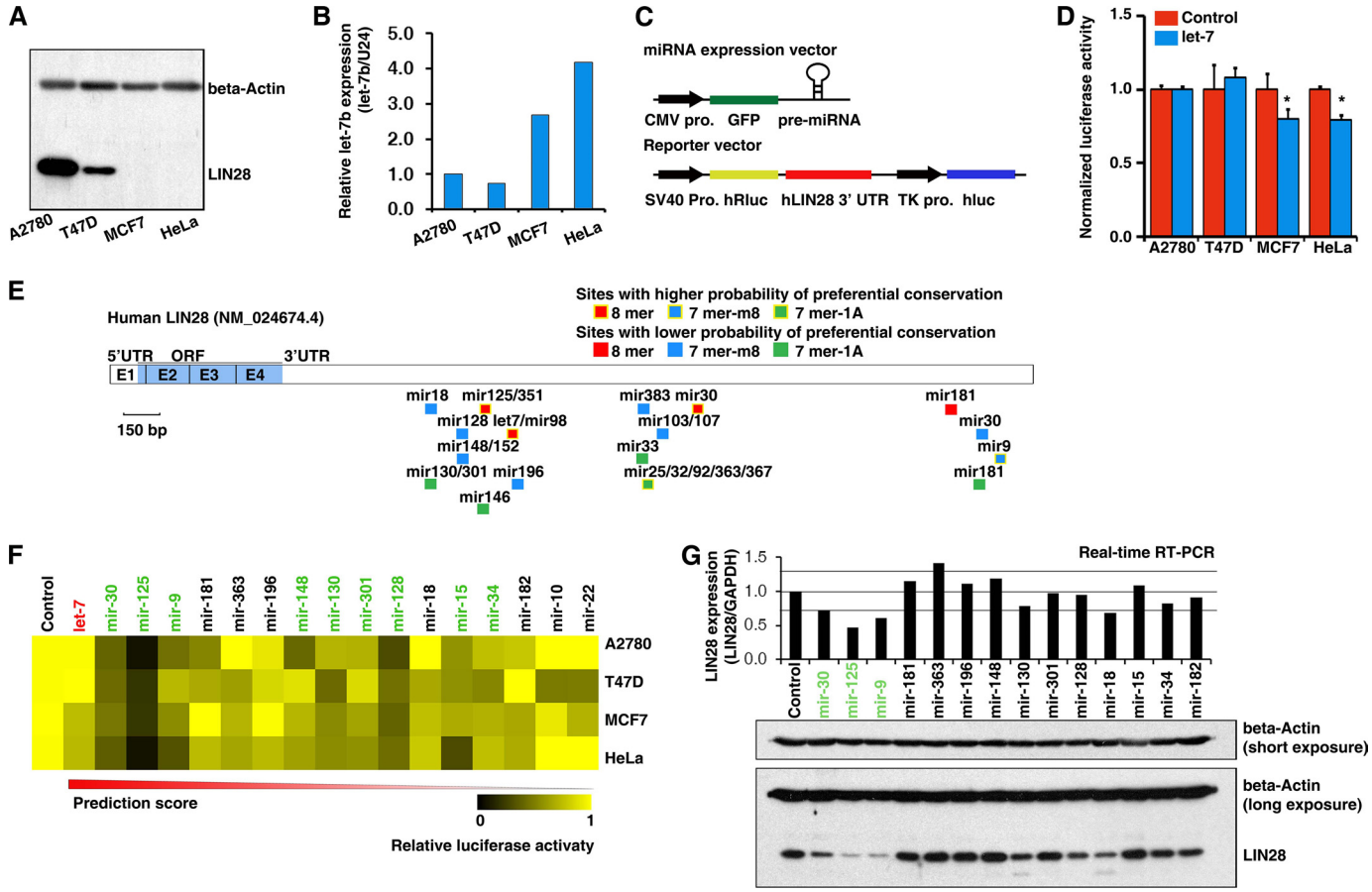


FIGURE 1. **A combined *in silico* prediction and miRNA library screening approach identified miRNAs targeting *LIN28*.** *A*, Western blots were used to detect endogenous LIN28 expression in the four cancer cell lines selected for experimental screening. Two of these (A2780 and T47D) were LIN28-positive, and two (MCF7 and HeLa) were LIN28-negative. *B*, real-time RT-PCR was used to detect *let-7b* expression in these cell lines. As expected, the LIN28-negative lines expressed relatively higher levels of *let-7b*. *C*, illustration of the miRNA expression vector and reporter vector used in the screening assay. *Pro.*, promoter; *hRluc*, *Renilla* luciferase; *hluc*, firefly luciferase. *D*, the known *LIN28*-regulatory miRNA *let-7* was used for the pilot screening. Co-transfection with the *let-7b* expression vector significantly (*, $p < 0.05$) reduced the luciferase activity of the *LIN28* 3′-UTR reporter vector in the two LIN28-negative cell lines. *E*, schematic structure of *LIN28* mRNA. The miRNA binding sites were predicted by TargetScan. *UTR*, untranslated region; *ORF*, open reading frame; *E*, exon. *F*, the summary heat map of the miRNA library screening in four cell lines. Here, miRNAs are listed from *left* to *right* according to their prediction scores (high to low). Nine miRNAs (marked in *green*) significantly reduced the reporter activity in all four cancer cell lines. *G*, stable cell lines overexpressing each of these nine miRNAs as well as five miRNA controls that did not reduce luciferase activity were generated by lentiviral infection. Three of the nine candidate miRNAs (*mir-30*, *mir-125*, and *mir-9*) markedly reduced both *LIN28* mRNA and protein expression.

the above miRNAs were selected from our genome-wide miRNA library. A pilot single transfection for each vector was performed in cancer cell lines, and enforced miRNA expression was confirmed by RT-PCR (data not shown). The miRNA vectors that were not efficiently expressed were excluded from the co-transfection screening. Finally, a total of 17 miRNAs were successfully used for the functional screen in all four cell lines. Briefly, nine of these miRNAs significantly reduced luciferase activity in all four cell lines (marked as *green* in Fig. 1*F*). To further test whether these 9 candidate miRNAs led to reduced expression of endogenous *LIN28*, we generated stable cell lines that overexpressed each of these nine miRNAs individually as well as stable cell lines expressing five miRNAs (as controls) that did not reduce luciferase activity. We found that three of the nine candidate miRNAs (*mir-30*, *mir-125*, and *mir-9*) markedly reduced both *LIN28* mRNA and protein expression (Fig. 1*G*). The direct regulation of two miRNAs in the *LIN28* 3′-UTR (*mir-9* and *mir-30*, which were first identified in our study) were confirmed by a mutant *LIN28* 3′-UTR reporter assay (Fig. 2). The seeding sequences of *mir-9* or *mir-30* in the *LIN28* 3′-UTR were mutated, and overexpression of *mir-9* or *mir-30* was shown to significantly reduce luciferase activity in the wild type but not the binding site mutant *LIN28* 3′-UTR reporters. Although *mir-18* decreased *LIN28* expression at a level comparable with that of *mir-30* (Fig. 1*G*), overexpression of *mir-18* was not able to reduce luciferase activity in the wild type and the binding site mutant *LIN28* 3′-UTR reporters in both A2780 and HeLa cells (data not shown). Taken together, four miRNAs, *let-7*, *mir-30*, *mir-125*, and *mir-9*, were identified in our initial screening. Importantly, two miRNAs known to regulate *LIN28* in mammalian (*let-7* and *mir-125*) (7, 61) were successfully identified using our approach.

miRNAs Regulate Lin28 in Undifferentiated ES Cells—Next, we examined whether the above miRNAs identified in cancer cell lines were able to regulate *Lin28* expression under physiological conditions, such as in undifferentiated ES cells. To address this question, we transiently transfected the mouse ES cell line R1 with the mimics of *let-7*, *mir-125*, *mir-9*, and *mir-30* and a control mimic. At 24 and 48 h post-transfection,

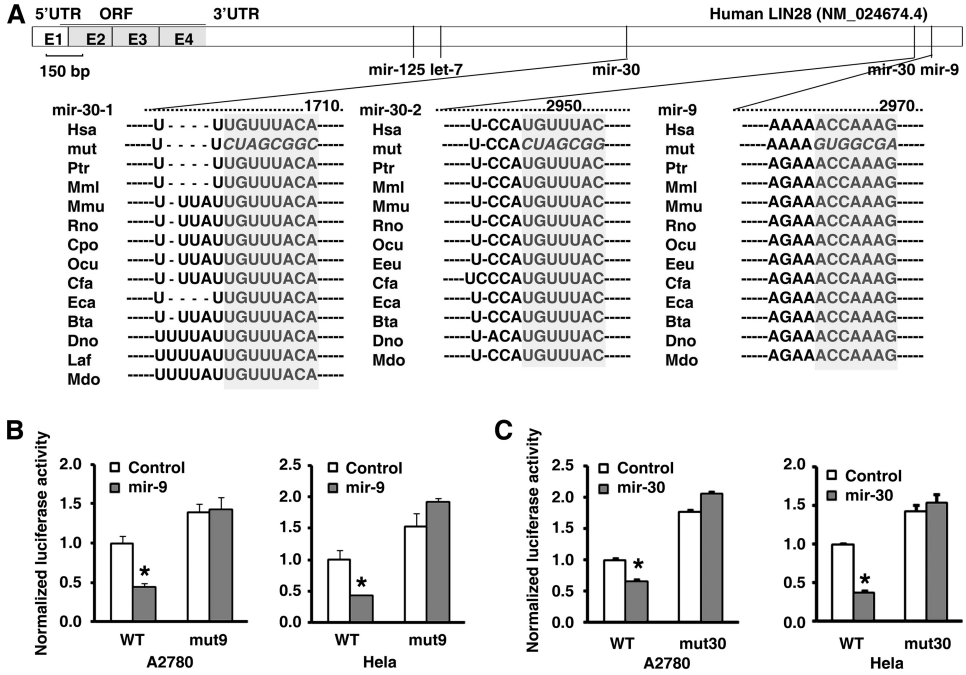


FIGURE 2. ***LIN28*-regulatory function of *mir-9* and *mir-30* was validated by the 3′-UTR reporter assay.** *A*, schematic diagram of the *mir-125*, *let-7*, *mir-30*, and *mir-9* binding sites in the *LIN28* 3′-UTR. The seeding sequences (marked in *gray*) were broadly conserved among different species. *Hsa*, Human; *Ptr*, chimpanzee; *Mml*, Rhesus; *Mmu*, mouse; *Rno*, Rat; *Cpo*, Pig; *Ocu*, rabbit; *Eeu*, Hedgehog; *Cfa*, Dog; *Eca*, Horse; *Bta*, Cow; *Dno*, armadillo; *Laf*, elephant; *Mdo*, opossum. *B* and *C*, summary of the reporter assays on the wild type and *mir-9* binding site mutant reporter (*B*) and *mir-30* binding site mutant reporter (*C*) in A2780 (LIN28-positive) and HeLa (LIN28-negative) cells. *WT*, wild type *LIN28* 3′-UTR reporter; *mut9*, *mir-9* binding site mutant *LIN28* 3′-UTR reporter; *mut30*, *mir-30* binding site mutant *LIN28* 3′-UTR reporter. Overexpression of *mir-9* or *mir-30* was able to significantly (*, $p < 0.05$) reduce luciferase activity in the wild type but not the binding site mutant *LIN28* 3′-UTR reporters.

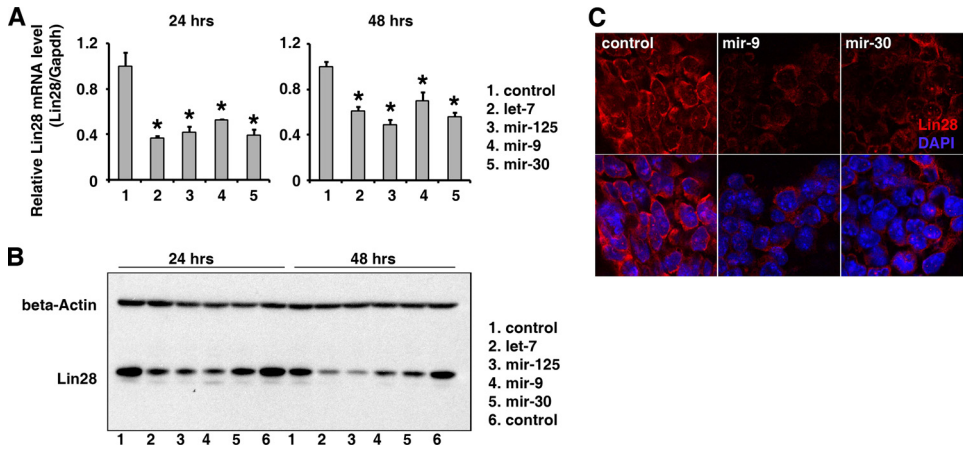
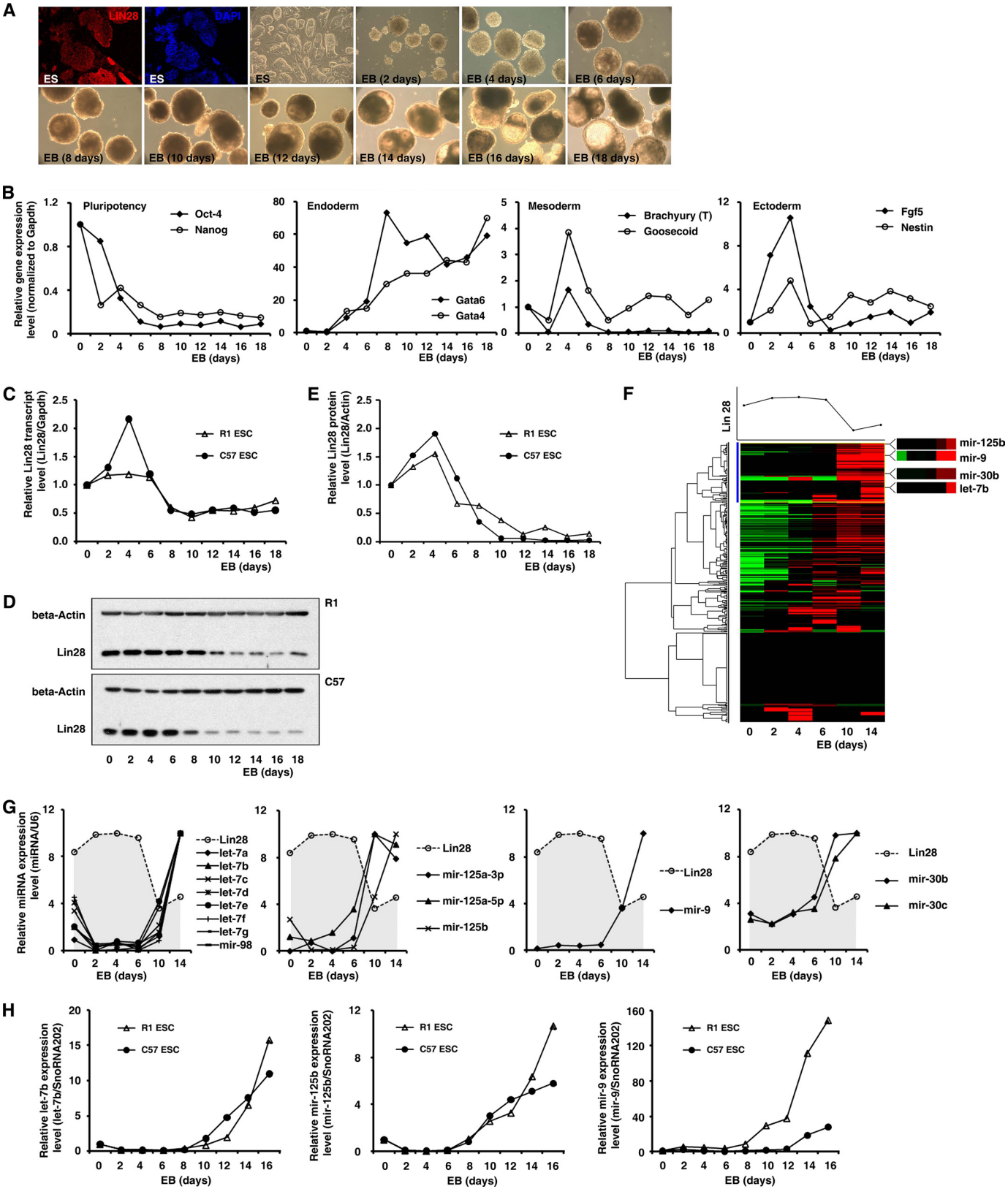


FIGURE 3. **miRNAs regulate *Lin28* in undifferentiated ES cells.** The miRNA mimics (30 nM) of *let-7*, *mir-125*, *mir-9*, *mir-30*, and a control mimic were transiently transfected into the mouse ES cell line R1. At 24 and 48 h post-transfection, total RNA and protein were collected, and the endogenous *Lin28* expression was examined by real-time RT-PCR (*A*) and Western blots (*B*). *A*, the *Lin28* mRNA expression was significantly (*, $p < 0.05$) decreased in the cells transfected with miRNA mimics compared with the control transfections. *B*, Lin28 protein expression was markedly decreased in the cells transfected with miRNA mimics compared with the control transfections. *C*, immunohistochemical staining further confirmed that *mir-9* and *mir-30* decreased LIN28 expression in ES cells.

total RNA and protein were collected, and the endogenous *Lin28* expression was examined by RT-PCR and Western blots. We found that the expression of both *Lin28* protein and mRNA was significantly decreased in the cells transfected with miRNA mimics compared with the control transfections (Fig. 3, *A* and *B*). For the two miRNAs (*mir-9* and *mir-30*) that were first identified as *LIN28* regulators in this study, we also confirmed the protein expression and localization by immunohistochemical staining. Consistent with the Western blot results, both *mir-9* and *mir-30* led to a reduction of endogenous *Lin28* in ES cells, but they had no effect on the cellular distribution of *Lin28* in these cells (Fig. 3*C*).

Finally, we asked whether the effects of the four miRNA families on *LIN28* expression are cumulative or if the effect of one miRNA is dominant over the others during ES cell differentiation. We transfected undifferentiated ES cells with each individual miRNA as well as a combination of all four miRNAs. We found that *mir-125* more efficiently suppressed *LIN28* expression compared with the other three miRNAs (supplemental Fig. S1*A*) and that the transfection of all four

miRNAs combined had a similar efficiency on the suppression of *LIN28* expression as *mir-125* alone (supplemental Fig. S1A). Serial combination transfections further demonstrated that *mir-125* was indeed the major functional miRNA in the combination transfection and that the effect of *mir-125* is dominant on *LIN28* expression when these miRNA are present in equal concentrations in ES cells (supplemental Fig. S1*B*). However, the mature miRNA expression levels during



ES cell differentiation will be another important factor affecting *LIN28* expression. To address this question, we retrieved previously published miRNA profiling data that was obtained by deep sequencing of small RNA libraries during ES cell differentiation (62). In differentiated ES cells, *mir-125a/b* yielded 1,557 sequence reads, and *let-7a/b/c/d/e/f/g/i*, *mir-9*, and *mir-30a/b/c/d/e* yielded 669, 2, and 4,284 sequence reads, respectively (supplemental Fig. S1*C*). We transfected the four miRNAs in the above ratios into undifferentiated ES cells in which all four miRNAs were absent. Consistently, we found that *mir-125* was most efficient at suppressing endogenous *LIN28* expression (supplemental Fig. S1*D*). Based on the above results, we conclude that the *mir-125* family is the most dominant miRNA regulating *LIN28* expression during ES cell differentiation, and that the *let-7* and *mir-30* families may also play important roles in the suppression of *LIN28* expression in the early stages of differentiation. Because the transfection of miRNA mimics only transiently affects miRNA expression, it is technically difficult to manipulate the levels of miRNAs throughout the entire process of ES cell differentiation. Therefore, to address the same question at later stages of ES cell differentiation, we retrieved three published miRNA profiling data sets from ES cell differentiation studies (63–65). We found that during ES cell differentiation, *let-7* is ubiquitously up-regulated/expressed in most somatic progenitor cells, whereas the other three miRNAs are probably tissue type-specific. For example, during the differentiation of ES cells to neurons, *let-7* is remarkably up-regulated, and *mir-9* is also significantly increased (63–65). This suggests that during the late differentiation stages in ES cells, these four miRNAs may serve distinct functions in different tissue lineages and that one or two of them may be more dominant at suppressing *LIN28* expression.

Expression Levels of LIN28 and Its Regulatory miRNAs Are Inversely Correlated during ES Cell Differentiation—Expression of *LIN28* is highly restricted to ES cells as well as developing tissues, and its expression dramatically decreases as differentiation proceeds (2, 4, 5, 8, 14, 27–29). Therefore, we examined how the expression levels of *LIN28* and its regulatory miRNAs dynamically changed during ES cell differentiation. ES cells are commonly differentiated *in vitro* by spontaneously self-assembling in suspension culture into three-dimensional cell aggregates called EBs, which model many of the hallmarks of early embryonic development. We chose two mouse ES cell lines (R1 and C57) and induced them to differentiate and form EBs (Fig. 4*A*). The germ layer maker expression patterns during EB formation (days 0–18) were monitored by real-time RT-PCR (Fig. 4*B*), and the expression of *Lin28* was examined by real-time RT-PCR and Western blots. *Lin28* mRNA levels were markedly decreased at day 6 of ES differentiation and EB formation (Fig. 4*C*), and *LIN28* protein expression was reduced at days 6–8 of differentiation (Fig. 4, *D* and *E*). To examine the global miRNA expression changes during EB formation, a low density TaqMan assay containing 381 miRNA probes was used (Fig. 4*F*). Interestingly, unsupervised cluster analysis indicated that all of the above four miRNA families (*let-7*, *mir-125*, *mir-9*, and *mir-30*) identified by our screening were clustered in the same group and shared similar expression patterns (Fig. 4*F*, *blue cluster*). Importantly, their expression levels were nearly non-detectable in undifferentiated ES cells and remarkably increased from day 6 at the same time that *Lin28* expression was decreased. Their expression levels then remained high in the differentiated EB cells (Fig. 4, *F* and *G*). This high throughput result was further validated by real-time RT-PCR assays in the two ES cell lines (Fig. 4*H*). In summary, during ES cell differentiation and EB formation, the expression level of *Lin28* decreases, whereas its regulatory miRNAs (*let-7*, *mir-125*, *mir-9*, and *mir-30*) display an inversely correlated expression pattern. This result strongly suggests that these four miRNAs play a functional role in *Lin28* regulation under physiological conditions such as early development.

LIN28-regulatory miRNAs Are Globally Down-regulated in LIN28-positive Cancer Cell Lines—*LIN28* has also been identified as a putative oncogene up-regulated/reactivated in 5–15% of human tumors (30–37). We examined whether the *LIN28*-regulatory miRNAs were also deregulated in these tumors. First, we reanalyzed a publicly available miRNA data set containing 218 specimens (normal control tissues, $n = 46$; tumors, $n = 172$) (66), and we successfully retrieved 16 miRNAs from the four *LIN28*-regulatory miRNA families. Interestingly, we found that 11 of these 16 miRNAs were markedly down-regulated (decreased more than 20%) in human tumors (Fig. 5, *A* and *B*). This indicates that the *LIN28*-regulatory miRNAs are deregulated in cancer. To provide further experimental evidence of this, we chose two cancer cell lines (A2780 and T47D) that highly express *LIN28* as well as 17 cancer cell lines in which *LIN28* protein was not detectable (Fig. 5*C*). The expression of *LIN28*-regulatory miRNAs was analyzed in these cell lines by real-time RT-PCR. As shown in Fig. 5*C*, all four miRNA families (*let-7*, *mir-9*, *mir-30*, and *mir-125*) displayed lower levels of expression in the LIN28-positive lines compared with the LIN28-negative lines. The negative correlation of *LIN28* expression with its suppressing miRNAs suggests that the global deregulation of these miRNAs may be an important mechanism of oncogenic *LIN28* up-regulation/reactivation in human cancer.

Next, we tested whether miRNA inhibitors were able to rescue/up-regulate endogenous *LIN28* expression in tumor cells. We transfected each individual miRNA inhibitor and a

FIGURE 4. **Expression levels of *Lin28* and its regulatory miRNAs are inversely correlated during ES cell differentiation.** *A*, ES cells were differentiated *in vitro* by spontaneously self-assembling in suspension culture into three-dimensional cell aggregates (EBs). Immunostaining demonstrated that Lin28 was highly expressed in the undifferentiated ES cells. *B*, pluripotency and differentiation markers during differentiation and EB formation were monitored by real-time RT-PCR. *C*, *Lin28* mRNA expression during EB formation was analyzed by real-time RT-PCR. *D* and *E*, Lin28 protein expression during EB formation was analyzed using Western blots. *F*, the global miRNA expression profile during EB formation was analyzed using a TaqMan miRNA assay. An unsupervised cluster analysis indicated that all four *Lin28*-regulatory miRNAs grouped together. *G*, detailed miRNA expression changes during EB formation identified by the TaqMan miRNA assay. All four *Lin28*-regulatory miRNAs were markedly up-regulated from day 6 of EB formation. *H*, real-time RT-PCR validations of the TaqMan miRNA assay in two ES cell lines.

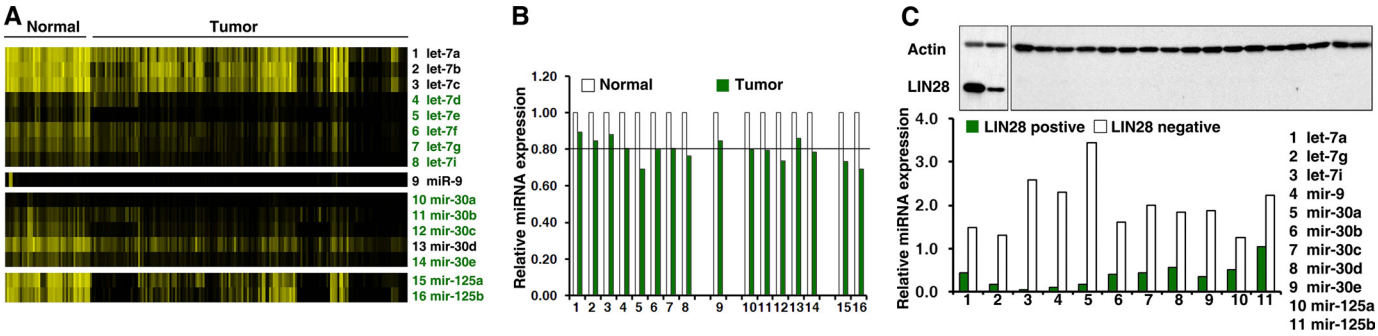


FIGURE 5. ***LIN28*-regulatory miRNAs are globally down-regulated in *LIN28*-positive cancer cell lines.** *A*, a publicly available miRNA microarray data set was retrieved from the Broad Institute (66). Normalized expression levels of miRNAs regulating *LIN28* were analyzed and are shown as a heat map. We found that 11 of 16 *LIN28*-regulatory miRNAs (marked in *green*) were markedly down-regulated (more than 20% reduction) in human tumors compared with normal control tissues. *B*, summary of the average expression levels of the miRNAs regulating *LIN28* in normal and tumor specimens in the public miRNA microarray data set. *C*, LIN28 protein levels in 19 human cancer cell lines detected by Western blot. Two of these cell lines (10.5%) were LIN28-positive. The *LIN28*-regulatory miRNA expression levels were analyzed by real-time RT-PCR in these 19 cell lines. Shown is a summary of the average expression of each individual miRNA in the LIN28-positive lines (*green*) and LIN28-negative lines (*white*).

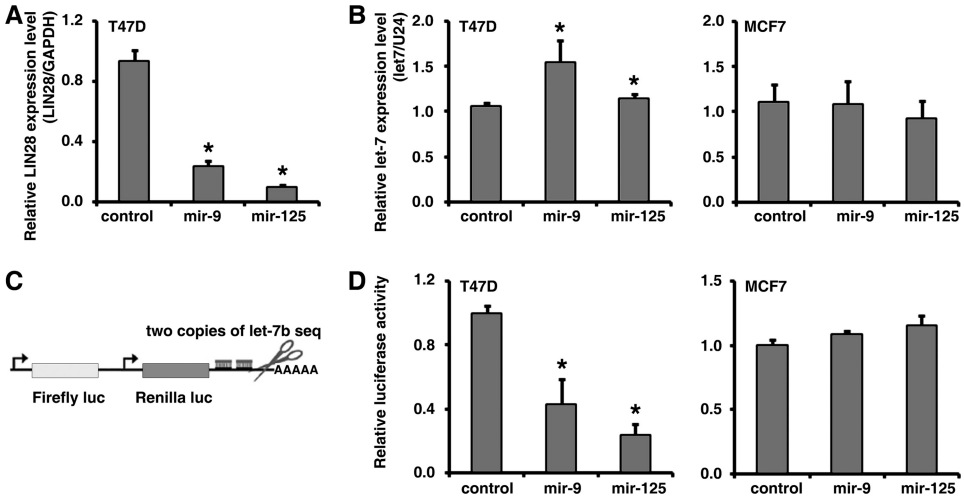


FIGURE 6. **miRNAs regulate *let-7* expression mediated by *LIN28*.** *A*, the transfection of *mir-9* and *mir-125* mimics significantly (*, $p < 0.05$) reduced *LIN28* mRNA expression in T47D cells. *B*, transfection of *mir-9* and *mir-125* mimics significantly (*, $p < 0.05$) increased *let-7b* expression in the LIN28-positive cell line T47D but not in the LIN28-negative cell line MCF7. *C*, a miRNA-responsive sensor, a technique for monitoring miRNA activity, bearing sequences complementary to *let-7b* in the downstream region of the 3′-UTR of a constitutively expressed reporter gene. *D*, transfection of *mir-9* and *mir-125* mimics significantly (*, $p < 0.05$) decreased the *let-7b*-responsive sensor in the LIN28-positive cell line T47D but not in the LIN28-negative cell line MCF7.

combination of these inhibitors as well as control oligonucleotides to both LIN28-positive (T47D) and LIN28-negative (HeLa) cell lines. We found that in the LIN28-positive cell line T47D, all four inhibitors, both individually and in a combination, were able to increase LIN28 protein expression. However, in the LIN28-negative cell line HeLa, none of these activated LIN28 expression to detectable levels (supplemental Fig. S2). Blocking all four miRNAs did not activate endogenous *LIN28* expression in LIN28-negative tumor cells. This suggests that the silencing of *LIN28* in adult tissues may be controlled by multiple mechanisms and not only by miRNA suppression. In LIN28-positive tumors, there may be at least two *LIN28*-suppressing pathways that are not functioning (*e.g.* miRNA suppression and epigenetic silencing).

miRNAs Regulate let-7 Expression Mediated by LIN28—Our finding suggested a novel regulatory mechanism in which one miRNA may indirectly regulate another miRNA via a protein coding gene; for example, *mir-125*, *mir-9*, and *mir-30* could regulate *let-7* expression via repression of *LIN28* expression. To test this hypothesis, we transfected *mir-9* and *mir-125* mimics into a LIN28-positive cell line (T47D). As expected, both *mir-9* and *mir-125* significantly reduced endogenous *LIN28* expression as detected by real-time RT-PCR (Fig. 6*A*). We then examined endogenous *let-7b* expression by real-time RT-PCR. As shown in Fig. 6*B*, both *mir-9* and *mir-125* significantly increased *let-7b* expression in T47D cells. Importantly, when we transfected *mir-9* or *mir-125* into a LIN28-negative cell line (MCF7), the *let-7b* expression levels were not affected (Fig. 6*B*). Finally, in order to monitor *let-7* activity, a *let-7* sensor (Fig. 6*C*) was co-transfected with the *mir-9* and *mir-125* mimics. As shown in Fig. 6*D*, *mir-9* and *mir-125* significantly reduced the luciferase activity of the *let-7* sensor in T47D cells, indicating that the endogenous *let-7* activity was increased by the *mir-9* and *mir-125* mimics. In the LIN28-negative line MCF7, the mimic transfection consistently did not change the activity of the *let-7* sensor. Taken together, our results demonstrate a novel regulatory mechanism where *LIN28*-regulatory miRNAs regulate *let-7* expression and activity via the protein-coding gene *LIN28*.

DISCUSSION

It is estimated that the human genome contains ~1,000 miRNAs (67), more than 900 of which have already been identified, according to the latest version of miRBase. miRNAs are predicted to target up to one-third of human mRNAs (60). Each miRNA can target hundreds of transcripts (68–71) and proteins (70, 71) directly or indirectly, and more than one miRNA can converge on a single transcript target (72). Therefore, the potential regulatory circuitry afforded by miRNA is enormous, but identification of miRNAs regulating protein-coding genes still remains challenging. Both bioinformatic prediction methods and whole genome-wide genetic screening have been used to characterize miRNA targets (58, 59). Here, using a combinatorial approach, we successfully identified four miRNA families, including two newly identified families (*mir-9* and *mir-30*), which regulate *LIN28* expression in ES cells and cancer cells. However, similar to the results obtained when miRNA library screening has been performed on other protein coding genes (58, 59), many predicted *LIN28*-regulatory miRNAs showed no significant effect on endogenous *LIN28* expression. For example, using a whole genome-wide miRNA library, Sage *et al.* (58) reported that only the *mir-221*/*mir-222* family regulated $p27^{Kip1}$, and Nagel *et al.* (59) also found that only the *mir-135* family suppressed *APC* expression. This suggests that protein-coding genes may be regulated by a smaller number of miRNAs than has previously been thought. However, in this study, we only included miRNAs that were predicted to bind to the *LIN28* 3′-UTR in our screening. Therefore, it is possible that other miRNAs may target other regions of *LIN28*. All four miRNA families identified in our study contain multiple members (*e.g.* the human *let-7* family contains 12 members; the *mir-125* family and the *mir-9* family each contain three members; and the *mir-30* family contains six members) (Table 2). These families are located in distinct chromosomal locations, suggesting that their expression is regulated by different 5′-UTR regulatory sequences and transcription factors. In addition, several *LIN28*-regulatory miRNAs are located in the same genomic loci and clustered together (*e.g. let-7e* and *mir-125a* are located in the same genomic locus, as are *mir-30b* and *mir-30d*) (Table 2). This suggests that they may be processed from the same primary miRNA transcript, and could coordinately regulate *LIN28* expression. Therefore, the potential regulatory circuitry afforded by these four miRNA families (at least 24 members) on *LIN28* is extensive and complex.

Expression of *LIN28* is highly restricted to ES cells as well as developing tissues and dramatically decreases as differentiation proceeds (2, 4, 5, 8, 14, 27–29). We found that the expression of all four miRNA families that we identified in this study was nearly undetectable in undifferentiated ES cells as well as in the first 6 days of EB formation and differentiation. During this time window, *LIN28* is expressed at a remarkably high level. From day 6 of EB formation, all four *LIN28*-regulatory miRNA families display a significantly increased expression, whereas both *LIN28* mRNA and protein levels begin to dramatically decrease (Figs. 4 and 7). This inversely related expression pattern suggests that these miRNAs may play an important functional role in the suppression of *LIN28* expression during development and differentiation. In most adult tissues, *LIN28* is almost completely silenced; however, these four miRNAs are expressed at high levels (Figs. 5*A* and 7). Interestingly, some of these miRNAs (*e.g. mir-9*) appear to be

TABLE 2
Genomic location and organization of the LIN28 regulatory miRNAs in humans

miRNA name	Cluster	Genomic location
mir-30e	*mir-30e* and *mir-30c-1*	1: 41220027–41220118 (+)
mir-30c-1	*mir-30c-1* and *mir-30e*	1: 41222956–41223044 (+)
mir-30c-2		6: 72086663–72086734 (−)
mir-30d	*mir-30b* and *mir-30d*	8: 135817119–135817188 (−)
mir-30a		6: 72113254–72113324 (−)
mir-30b	*mir-30b* and *mir-30d*	8: 135812763–135812850 (−)
let-7a-1	*let-7a-1*, *let-7f-1*, and *let-7d*	9: 96938239–96938318 (+)
let-7a-2	*mir-100* and *let-7a-2*	11: 122017230–122017301 (−)
let-7a-3	*let-7a-3* and *let-7b*	22: 46508629–46508702 (+)
let-7b	*let-7a-3* and *let-7b*	22: 46509566–46509648 (+)
let-7c	*mir-99a* and *let-7c*	21: 17912148–17912231 (+)
let-7d	*let-7a-1*, *let-7f-1*, and *let-7d*	9: 96941116–96941202 (+)
let-7e	mir-99b, let-7e and mir-125a	19: 52196039–52196117 (+)
let-7f-1	*let-7a-1*, *let-7f-1*, and *let-7d*	9: 96938629–96938715 (+)
let-7f-2	*let-7f-2* and *mir-98*	X: 53584153–53584235 (−)
let-7g		3: 52302294–52302377 (−)
let-7i		12: 62997466–62997549 (+)
mir-98	*let-7f-2* and *mir-98*	X: 53583184–53583302 (−)
mir-125a	*mir-99b*, *let-7e*, and *mir-125a*	19: 52196507–52196592 (+)
mir-125b-1		11: 121970465–121970552 (−)
mir-125b-2		21: 17962557–17962645 (+)
mir-9-1		1: 156390133–156390221 (−)
mir-9-2		5: 87962671–87962757 (−)
mir-9-3		15: 89911248–89911337 (+)

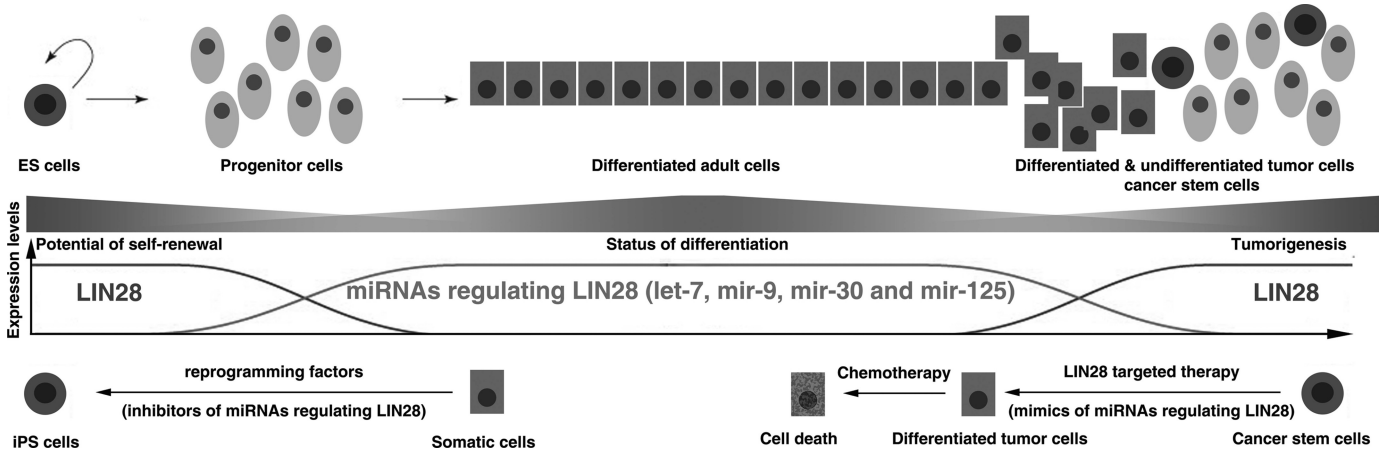


FIGURE 7. **The regulatory circuitry afforded by *LIN28*-regulatory miRNAs in development and tumorigenesis.**

tissue-specific, whereas some of them (*e.g. mir-30*) are expressed in nearly all adult tissues. This indicates that there is a complex temporal and spatial regulatory network of miRNAs affecting *LIN28* expression under physiological conditions. Finally, given that *LIN28* can act as a reprogramming factor together with *OCT4*, *SOX2*, and *NANOG* to reprogram somatic cells to induced pluripotent stem cells (19), the question arises as to whether co-transfection of *LIN28*-regulatory miRNA inhibitors with these reprogramming factors could increase the efficiency of reprogramming (Fig. 7). Supporting this hypothesis, Melton *et al.* (73) have recently reported that inhibition of *let-7* promotes dedifferentiation of somatic cells to induced pluripotent stem cells.

In human cancer, *LIN28* is up-regulated/reactivated in about 5–15% of patients and functions as an oncogene (30–37). Interestingly, we found that these four *LIN28*-regulatory miRNAs are globally down-regulated in tumors (Figs. 5 and 7), possibly due to genetic and/or genomic alterations in these miRNAs or deregulation of their biogenesis pathways (66). Notably, in the LIN28-positive tumor cell lines, these miRNAs were expressed at relatively lower levels compared with the LIN28-negative tumor cell lines. Therefore, down-regulation of *LIN28*-regulatory miRNAs may be an important mechanism of *LIN28* reactivation in human cancer (Fig. 5*C*). Recent studies have demonstrated that *LIN28* serves as an oncogene promoting malignant transformation and tumor growth (30–37). Importantly, *LIN28* may contribute to the maintenance of cancer stem cells, a relatively rare subpopulation of tumor cells having the unique ability to initiate and perpetuate tumor growth (34). Rapidly accumulating evidence indicates that miRNAs are involved in the initiation and progression of cancer (66, 74–80). Interestingly, two of the *LIN28*-regulatory miRNAs (*let-7* and *mir-125*) have been demonstrated as tumor suppressor genes. For example, the tumor suppressor role of *let-7* in cancer was first demonstrated by the Slack laboratory (76). It was found that the *let-7* family negatively regulates *let-60*/RAS in *C. elegans* by binding to multiple *let-7*-complementary sites in its 3′-UTR (76). Moreover, the finding that *let-7* expression is lower in lung tumors than in normal lung tissue, whereas RAS protein is significantly higher in lung tumors, proposes *let-7* as a tumor suppressor gene (76, 81–84). Therefore, our study could lead to new therapeutic strategies for cancer treatment (Fig. 7). For example, nanoparticle-delivered *LIN28*-regulatory miRNA mimics may be an attractive therapeutic method to target the LIN28-positive cancer stem cell population in tumors.

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