Extracellular Signal-regulated Kinases (ERKS) Phosphorylate Lin28a Protein to Modulate P19 Cell Proliferation and Differentiation*5

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Lin28a, originally discovered in the nematode Caenorhabditis elegans and highly conserved across species, is a well-characterized regulator of let-7 microRNA (miRNA) and is implicated in cell proliferation and pluripotency control. However, little is known about how Lin28a function is modulated at the post-translational level and thereby responds to major signaling pathways. Here we show that Lin28a is directly phosphorylated by ERK1/2 kinases at Ser-200. By editing lin28a gene with the CRISPR/Cas9-based method, we generated P19 mouse embryonic carcinoma stem cells expressing Lin28a-S200A (phospho-deficient) and Lin28a-S200D (phospho-mimetic) mutants, respectively, to study the functional impact of Ser-200 phosphorylation. Lin28a-S200D-expressing cells, but not Lin28a-S200A-expressing or control P19 embryonic carcinoma cells, displayed impaired inhibition of let-7 miRNA and resulted in decreased cyclin D1, whereas Lin28a-S200A knock-in cells expressed less let-7 miRNA, proliferated faster, and exhibited differentiation defect upon retinoic acid induction. Therefore our results support that ERK kinase-mediated Lin28a phosphorylation may be an important mechanism for pluripotent cells to facilitate the escape from the self-renewal cycle and start the differentiation process.

Lin28a (cell lineage abnormal 28), an RNA-binding protein highly conserved across eukaryotes from Caenorhabditis elegans to mammals, is an important regulator involved in various physiological processes including cell proliferation, differentiation, and organ development, as well as metabolism and homeostasis (1, 2). By directly binding to its target RNAs, Lin28a is known to inhibit maturation of let-7 miRNA2 family and promotes their turnover (3, 4), thereby influencing an array of let-7 targets including c-Myc, Ras, and cyclin D1, as well as Lin28a itself (5, 6), which are master regulators of cell proliferation and the pluripotent status of stem cells. Although Lin28a is also found directly bound to mRNAs of several important metabolic enzymes and influences the translation of these mRNAs (6–8), its function in stem cell differentiation and development is primarily dependent on let-7 miRNAs (3).

The Lin28a-let-7 axis has been implicated in neurogenesis. Briefly, during the development of the central nervous system, let-7 miRNAs quickly accumulate, and then silence target genes including pluripotency factors and fetal oncoproteins to drive the neural stem cells to differentiate (9, 10). As a consequence, the members of the let-7 family are among the most abundant miRNAs in adult brain. Such cell fate determination is a complex process and needs to be precisely coordinated with exit from cell cycle (11, 12), and therefore involves crosstalk between the molecular pathways controlling proliferation and differentiation. Because let-7 miRNAs are tightly governed by Lin28a in neural stem cells, a prompt mechanism is required to respond to environmental signal and attenuate the inhibitory effect of Lin28a. On the other hand, mitogen-activated protein kinase (MAPK) signaling pathway plays an important role in controlling cell proliferation in most somatic cells by facilitating the transition through early G1 phase of the cell cycle. Activation or prolongation of MAPK signaling often induces differentiation, then connects cell proliferation and development events (13, 14). Several studies have indicated that MAPK signaling promotes commitment to terminal differentiation and inhibits self-renewal in stem cells (15–18). MAPK inhibitors enhance self-renewal of mouse ES cells, and ERK2 null ES cells lose the ability to undergo differentiation (18). Interestingly, MAPK signaling has been indicated to modulate cyclin D1 mRNA levels during stem cell differentiation (19, 20), yet the mechanism remains elusive. Because cyclin D is the target of the Lin28a-let-7 axis, MAPK signaling may affect cell cycle/cell differentiation balance via modulating Lin28a.

Herein, we investigated the direct relationship between ERK kinases (MAPK1/3), the major downstream kinase of MAPK signaling, and Lin28a. Consistent with a very recent study (21), we first characterized Ser-200 of Lin28a as a putative ERK phosphorylation site. By using the mouse P19 embryonic carcinoma (EC) cell line (22, 23), an established tool for studying the molecular and cellular mechanism of self-renewing and differentiation in vitro (24–26), we generated Lin28a-S200A (phospho-deficient) and Lin28a-S200D (phospho-mimetic) knock-in cell lines. Our results revealed that Ser-200 phosphor-
self-renewal and differentiation.

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**Results**

**Lin28a Is Phosphorylated at Ser-200**—To investigate possible post-translational modification of Lin28, we performed transfection-based immunoprecipitation-mass spectrometry analysis and recovered only one phosphorylation of Lin28a at Ser-200 (Fig. 1A and supplemental Fig. S1A), which is highly conserved through evolution. A phospho-motif antibody (K/H)pSPX(K/R), where underlining indicates the phosphoserine from Cell Signaling Technology termed CS2 was utilized to detect Ser-200 phosphorylation of Lin28a-WT but not Lin28a-S200A mutant from both human and mouse origin. We also generated a phospho-specific antibody (pLin28a) that recognizes human or mouse Lin28a only when the Ser-200 residue is phosphorylated (Fig. 1, B and C, and supplemental Fig. S1B). We further validated Lin28a Ser-200 phosphorylation at the endogenous level in the P19 EC cell line, in which the function of Lin28a has been studied (see Refs. 27 and 28), but not in lin28a knock-out cells or Lin28a-S200A (phospho-deficient) knock-in cells generated by the CRISPR/Cas9-based gene editing method (Fig. 1D and supplemental Fig. S1C).

**ERK Kinases Phosphorylate Lin28a at Ser-200**—To identify the upstream signaling responsible for Ser-200 phosphorylation, we performed a kinase inhibitor screening. As indicated in Fig. 2A, MAPK-ERK pathway inhibitor AZD6244 and U0126, but not cyclin-dependent kinase inhibitor PD0332991, drastically abolished the Lin28a phosphorylation signal detected by both CS2 motif and pLin28a antibodies, respectively, indicating that MAPK-ERK pathway may control this phosphorylation. To further dissect whether Lin28a Ser-200 is phosphorylated by ERK1/2 or upstream kinases such as MEK and MEKK, we depleted ERK1/2 kinases with multiple shRNA constructs and examined the pLin28a signal. Interestingly, efficient knockdown of ERK1 or ERK2 all caused marked reduction of Ser-200 phosphorylation of ectopically expressed Lin28a, supporting that ERK1/2 may be the kinase modulating this phosphorylation (Fig. 2B). Consistently, Ser-200 phosphorylation of endogenous and exogenous Lin28a expressed in P19 cells was sensitive to MAPK inhibitors including AZD6244 and U0126 (Fig. 2, C and D), whereas co-expression of the constitutively active (CA) form of MEK1 or ERK1 kinase assay in vitro...
Lin28a Ser-200 Phosphorylation Impairs Its Inhibitory Effect upon let-7 and Affects P19 Cell Proliferation and Differentiation—

To investigate the physiological function of Lin28a Ser-200 phosphorylation, we generated P19 knock-in cells where the coding sequence for the Ser-200 residue of *lin28a* was replaced to express Lin28a-S200A (phospho-deficient) and Lin28a-S200D (phospho-mimetic) mutants, respectively (supplemental Fig. S3). Three independent colonies were selected for Lin28a-S200A and Lin28a-S200D knock-in P19 cells, and the sequences were validated by Sanger sequencing. Notably, Lin28a-S200A mutant, suggesting that ERK kinases directly phosphorylate Lin28a at Ser-200 site.

**FIGURE 2. Erk kinases mediates phosphorylation of Ser-200.**

A, HeLa cells were transfected with 1 μg of HA-h-Lin28a. 36 h after transfection, cells were pretreated with various kinase inhibitors before being harvested for HA immunoprecipitation (IP) and whole-cell lysates (Input). Then IP and input were detected by the indicated antibodies. MAPK-ERK inhibitors AZD6244 (10 μM) and U0126 (10 μM) and CDK4/6 inhibitor PD032991 (1 μM) were used as indicated. B, HeLa cells were co-transfected with 1 μg of HA-h-Lin28a and 3 μg of shRNAs against ERK1/2. 36 h after transfection, cells were harvested for HA immunoprecipitation (IP) and whole-cell lysates (Input). Then IP and input were detected by the indicated antibodies. C, immunoblotting (IB) analysis of endogenous Lin28a Ser-200 phosphorylation in P19 cells after treatment with various kinase inhibitors. P19 cells were serum-starved for 18 h before the addition of kinase inhibitors and 10% serum. 5 h later, cells were harvested to prepare whole-cell lysates and detected by the indicated antibodies. MAPK-ERK inhibitors AZD6244 (10 μM) and U0126 (10 μM) and CDK4/6 inhibitor PD032991 (1 μM) were used as indicated. D, P19 cells were co-transfected with 1 μg of mouse Lin28a constructs, wild type (HA-m-Lin28a) or phospho-deficient (HA-m-Lin28a-S200A), and with 2 μg of constitutively active HA-MEK1 (HA-MEK1 CA) construct. 36 h after transfection, cells were harvested for FLAG immunoprecipitation (IP) and whole-cell lysates (Input). Then IP and input were detected by the indicated antibodies. E, P19 and S200A (SA) knock-in cells were serum-starved for 18 h and then split into bacterial grade Petri dish with medium containing 10% serum, retinoic acid, and U0126 (10 μM) as indicated. 24 h later, cells were harvested and detected by the indicated antibodies. F, P19 and S200A knock-in cells were transfected using Lipofectamine with 2 μg of constitutively active HA-MEK1 construct. 24 h after transfection, cells were split into a bacterial grade Petri dish and induced with RA for another 24 h. Then cells were harvested and signal was detected by the indicated antibodies. G, ERK1 phosphorylates Lin28a Ser 200 in vitro. HA-ERK1 kinases were prepared by immunoprecipitation from 293T cells transfected with 2 μg of HA-ERK1 construct. 2 μg of wild type Lin28a or Lin28a-S200A recombinant proteins were incubated with HA-ERK1 kinase in the presence of ATP. 30 min later, the kinase reactions were stopped by the addition of the SDS sample buffer. The reaction products were resolved by SDS-PAGE, and phosphorylation of Lin28a was detected with CS2 motif antibody.
Significant increases of let-7a and let-7g miRNAs were detected in all three S200D knock-in colonies, whereas much lower amounts of let-7a and let-7g were expressed in all three S200A knock-in colonies, when compared with control P19 cells (Fig. 3, A and B). Therefore Ser-200 phosphorylation may impair the function of Lin28a in suppressing let-7 family miRNA. Consistently, we observed a sharp decrease of cyclin D1, which is a well known let-7 target gene, at both mRNA and protein levels in S200D knock-in cells. On the other hand, cyclin D1 was drastically increased in all three S200A knock-in colonies (Fig. 3, C and D).

To further investigate whether such a difference in let-7 miRNAs in S200D and S200A knock-in cells would cause any effect.
in cell division and differentiation, we performed a cell proliferation assay as well as RA treatment to induce differentiation. In line with cyclin D1 expression levels, S200A knock-in cells grew faster, whereas S200D knock-in cells grew slower than control P19 cells (Fig. 3E). Additionally, after induction with RA, the differentiation process in S200A knock-in cells was much slower when compared with control P19 cells and S200D knock-in cells, as evidenced by the relatively lower mRNA levels of neural marker *ngn1* and *mask1* (Fig. 3F). High expression of alkaline phosphatase (AP) is regarded as a feature of stem cells, so AP staining assays are widely used to evaluate cell pluripotent status. 4 days after RA treatment, S200A knock-in cells still had a much stronger AP staining signal when compared with P19 wild type cells and S200D knock-in cells, indicating that S200A knock-in cells indeed have a much slower differentiation program (Fig. 3G). Taken together, the results outlined above suggest that ERK-mediated Ser-200 phosphorylation is important in the inhibition of Lin28a function in P19 cells.

**Discussion**

As a conserved RNA-binding protein, Lin28a is expressed in pluripotent cells including EC cells such as P19, and plays key roles in maintaining self-renewal and promoting proliferation of stem cells by both *let-7*-dependent and *let-7*-independent mechanisms (1, 2, 4, 29). It has been reported that Lin28a can inhibit *let-7* via both TUT4/7-dependent and TUT4/7-independent mechanisms. In the cytoplasm, Lin28a recruits TUT4/7 and Trim25 to oligo-uridylate *pre-let-7* and promote their degradation (28, 30, 31). On the other hand, nuclear Lin28a can also bind *pri-let-7* in the nucleolus to prevent processing by Drosha (32). Because Ser-200 phosphorylation is not adjacent to the nuclear localization signal of Lin28a, it is less likely to affect Lin28a subcellular localization. Therefore both inhibitory mechanisms are possibly affected by Ser-200 phosphorylation of Lin28a. Our finding of ERK-dependent phosphorylation at Ser-200 of Lin28a provides a solid mechanism by which extracellular stimulation could promptly inhibit Lin28a function and increase *let-7* miRNA levels to suppress a wide range of gene expression, thus connecting Lin28a-*let-7* axis to well established MAPK kinase signaling pathway. We noticed that results in another recent study implicated a *let-7*-independent role of Lin28a Ser-200 phosphorylation (21), and we think that the discrepancy may be caused by the different experimental system used. We utilized mainly S200A and S200D knock-in cells derived from P19 EC cells, whereas the other study relied on ectopic expression of Lin28a in dH1f and PA1 cells to perform *let-7*-related experiments. However, our work and the other study both support an important regulatory function of Ser-200 phosphorylation of Lin28a.

Proliferation and differentiation are usually tightly coupled processes for stem cells. Decelerated cell cycle progression is often linked to the commitment of cell fate determination (11, 12, 33–35). Previously, cell cycle proteins of pluripotent cells were thought to control only the escape from the terminal cell cycle during differentiation, although their levels always remain in a state of flux. Recent studies support that cell cycle regulators, particularly cyclin D1, can also play a key role in the self-renewal versus commitment cell fate decision (36–40). MAPK-ERK pathway is widely accepted as the dominant signaling pathway driving cell cycle progression and has been indicated in cyclin D1 regulation (19). Our results demonstrating differential cyclin D1 expression in S200A and S200D knock-in cells indicate another layer of regulation that connects MAPK-ERK pathway to cyclin D1 through ERK kinase-mediated Ser-200 phosphorylation of Lin28a. Moreover, the defects of S200A knock-in cells in the RA-induced differentiation assay further suggested that activation of MAPK-ERK pathway is at least in part required for P19 cell differentiation through Lin28a (Fig. 3G).

In summary, our finding of ERK-mediated Ser-200 phosphorylation of Lin28a not only expands current understanding of regulatory mechanisms for Lin28a-*let-7* axis, but also provides new hints about how cytokines that activate MAPK-ERK pathway would affect gene expression profiles in physiological and pathological conditions.

**Experimental Procedures**

**Cell Culture**—P19 cells were cultured in DMEM/F12 (1:1) containing 10% FBS (Invitrogen). HeLa and HEK293T cells were cultured in DMEM containing 10% FBS (Invitrogen). All cells were cultured at 37 °C in 5% CO2 atmosphere. RA-induced P19 differentiation was performed as described previously (26). In brief, cells were plated in a bacterial grade Petri dish in media containing 5 × 10−7 m all-trans RA (Sigma) for 4 days. The cell aggregates were resuspended by trypsin treatment and then replated to a tissue culture dish.

**Plasmids and Chemical Reagents**—cDNAs of human Lin28a and mouse Lin28a were amplified by RT-PCR from the total RNA of H9 human ES cells and P19 EC cells, and the PCR products were inserted into pcDNA3.1-HA or pcMV-FLAG vectors. S200A and S200D point mutations were generated via site-directed mutagenesis and then validated by Sanger sequencing. Sequences for constructing shRNA targeting human ERK kinases were obtained from the RNAi Consortium (Broad institute), and shRNAs against target genes were generated with pLK0.1 vector (target sequences are shown in supplemental Tables S1-S3).

The constitutively active MEK1 (MEK1 CA) was constructed as described previously (41). sgRNA was constructed by inserting oligonucleotides into the pX330 vector (42). Small molecule inhibitors U0126, AZD6244, and PD0332991 were purchased from Selleck Chemicals (Houston, TX).

**Generation of Lin28a Point Mutation Knock-in P19 Cells**—lin28a S200A and S200D point mutation knock-in cells were generated by the CRISPR/Cas9 gene editing method. The sequences of sgRNA and ssODN are listed in the supplemental material. Briefly, 2 μg of sgRNA plasmid, 3 μg of ssODN oligonucleotide, and 0.1 μg of puro-resistant plasmid were co-transfected into P19 cells using Lipofectamine (Invitrogen). 2 days after transfection, cells were selected with 1 μg/ml puromycin for 2 days. Surviving cells were counted, and 200 cells were plated in a 100-mm dish. Cell colonies were selected after 10 days of further culturing. The genomic fragments of the Lin28a gene were first examined by PCR, and then DNA samples with the expected fragments were validated by Sanger sequencing.
**Immunoprecipitation (IP), Western Blotting, and Antibodies**—Cells were harvested with EBC lysis buffer (50 mM Tris HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40) supplemented with protease inhibitors (Selleck Chemicals) and phosphatase inhibitors (Selleck Chemicals). 50 μg of total proteins were separated by SDS-PAGE gel and blotted with primary antibodies. For IP, 800 μg of cell lysates were incubated with the indicated antibody (1–2 μg) for 3 h at 4 °C, and then protein A-Sepharose beads (GE Healthcare) were added into a mixture of cell lysates and antibody, and then incubated at 4 °C for another 1 h. IP complexes were washed 5 times with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). After washing, the IP samples were resolved by SDS-PAGE and immunoblotted with the appropriate antibody. The details about primary antibodies are listed below with vendor names and catalog numbers: Lin28a (Santa Cruz Biotechnology, SC293120), Lin28a (AbClonal, A6034), CS2 (CST, 9477), CCND1 (cyclin D1, Arigo, ARG52923), cyclin A (Santa Cruz Biotechnology, SC751), vinculin (Sigma, V4505), tubulin (Santa Cruz Biotechnology, SC23948), mouse anti-HA tag (Santa Cruz Biotechnology, SC7392), rabbit anti-HA tag (Santa Cruz Biotechnology, SC805), mouse anti-FLAG tag (Sigma, F3165S), rabbit anti-FLAG tag (Sigma, F7425S), pErk (CST, 4370), and Erk1/2 (Santa Cruz Biotechnology, SC93). pLin28a antibody was generated by AbClonal Biotechnology Co., Ltd. (Wuhan, China).

**RNA Isolation and Quantitative Real-time PCR**—Total RNA was prepared using TRIzol (Invitrogen), and was reverse-transcribed by PrimeScript RT Reagent Kit with genomic DNA (gDNA) Eraser (Takara, RR047A). The qPCR reactions were run on an ABI Q6 real-time PCR instrument. Levels of let-7a and let-7g miRNAs were detected by TaqMan MicroRNA Assay (ABI Scientific) and normalized by U6 small nuclear RNA.

**Cell Growth Experiment**—To determine the cell growth curve, 2 × 10⁵ cells were plated in 60-mm dishes, and cells were counted daily. Media were changed every day.

**In Vitro Kinase Assay**—293T cells were transfected with HA-ERK1. 48 h after transfection, cells were harvested, and HA-ERK1 was immunoprecipitated with HA matrix (Roche Diagnostics). Then 5 μg of His-Lin28a proteins (wild type or S200A mutant) were incubated with HA-ERK1 in the presence of ATP and in vitro kinase buffer. The reactions were stopped by the addition of SDS-PAGE loading buffer, and the products were resolved by SDS-PAGE and immunoblotted by the indicated antibodies.

**AP Staining**—Cells were fixed with 4% paraformaldehyde at room temperature for 10 min and stained for alkaline phosphatase using an Alkaline Phosphatase Color Development Kit (Beyotime Biotechnology) according to the manufacturer’s instructions.

**Author Contributions**—X. L., M. C., I. L., and L. G. conducted the experiments. D. G. supervised the project. X. L., M. C., I. L., L. G., and H. Z. analyzed the data. D. G. wrote the manuscript with input from the other authors.

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