

# *Lin28* regulates the expression of neuropeptide Y receptors and oocyte-specific homeobox genes in mouse embryonic stem cells

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**Objective:** *Lin28* has been known to control the proliferation and pluripotency of embryonic stem cells. The purpose of this study was to determine the downstream effectors of *Lin28* in mouse embryonic stem cells (mESCs) by RNA interference and microarray analysis.

**Methods:** The control siRNA and *Lin28* siRNA (Dharmacon) were transfected into mESCs. Total RNA was prepared from each type of transfected mESC and subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis to confirm the downregulation of *Lin28*. The RNAs were labeled and hybridized with an Affymetrix Gene-Chip Mouse Genome 430 2.0 array. The data analysis was accomplished by GenPlex 3.0 software. The expression levels of selected genes were confirmed by quantitative real-time RT-PCR.

**Results:** According to the statistical analysis of the cDNA microarray, a total of 500 genes were altered in *Lin28*-downregulated mESCs (up-regulated, 384; down-regulated, 116). After differentially expressed gene filtering, 31 genes were selected as candidate genes regulated by *Lin28* downregulation. Among them, neuropeptide Y5 receptor and oocyte-specific homeobox 5 genes were significantly upregulated in *Lin28*-downregulated mESCs. We also showed that the families of neuropeptide Y receptor (*Npyr*) and oocyte-specific homeobox (*Obox*) genes were up-regulated by downregulation of *Lin28*.

**Conclusion:** Based on the results of this study, we suggest that *Lin28* controls the characteristics of mESCs through the regulation of effectors such as the *Npyr* and *Obox* families.

**Keywords:** *Lin28*; Embryonic stem cells; RNA interference; Microarray; Neuropeptide Y receptors; Oocyte-specific homeobox

## Introduction

Embryonic stem cells (ESCs) derived from the inner cell mass of blastocyst-stage embryos have two essential abilities, self-renewal and

pluripotency [1]. Self-renewal allows them to persist in an environment preventing their differentiation, and pluripotency enables them to differentiate and form all three embryonic germ layers. Studies have been performed on the control mechanisms of such capabilities of ESCs, and it has been found that four genes - *Oct4*, *Sox2*, *Klf4*, and *c-Myc* - are responsible for making the induced pluripotent stem (iPS) cells when the genes are overexpressed in somatic cells [2-4]. The iPS cells have been shown to be pluripotent and similar to ESCs, both in their markers and in their gene expression. In addition, other genes, *Lin28* and *Nanog*, were also shown to facilitate the derivation of iPS cell formation in combination with *Oct4* and *Sox2* [3,5].

*Lin28* is an RNA binding protein and originally identified as a heterochronic gene in controlling the developmental timing of *Caenorhabditis elegans* [6]. However, the molecular mechanism by which *Lin28* controls developmental timing is still unclear. In mammals, *Lin28* is

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widely expressed in early-stage embryos, with expression decreasing and becoming restricted to a few tissues such as skeletal and cardiac muscles [7]. In human tissues, *Lin28* has been detected in normal ovarian surface epithelium and in mature oocytes [8]. In human and mouse ESCs, in particular, *Lin28* is abundantly expressed and decreases dramatically upon induction of differentiation [9,10], and *Lin28* is involved in the cell proliferation and facilitates the expression of *Oct4* at the post-transcriptional level [11,12].

The biological significance of *Lin28* is spotlighted by its capability to enhance the reprogramming of fibroblasts to iPS cells by the replacement of other factors such as *Klf4* and *c-Myc* [3]. In addition, *Lin28* is specifically activated in the subset of tumors that are poorly differentiated and associated with the worst prognosis [13]. *Lin28* is predominantly located in the cytoplasm and plays roles in the regulation of gene expression in normal tissues and cancers. *Lin28* has been suggested to be a post-transcriptional regulator by its RNA binding ability and has been shown to block let-7 microRNA (miRNA) processing by binding the loop of miRNA [14,15]. Therefore, *Lin28* has an ability to promote reprogramming and maintain the self-renewal of ESCs by preventing production of mature let-7 miRNAs [16]. Also, this mechanism has been implicated in the aggressiveness of many different tumors by activating *Lin28* protein [13]. *Lin28* has also been reported to bind a specific subset of mRNAs and modulate their expression in addition to blocking the let-7 miRNA processing [17].

Based on the fact that *Lin28* plays pleiotropic roles in the regulation of gene expression, in this study, we determined the downstream effectors regulated by *Lin28* in mouse embryonic stem cells (mESCs) using RNA interference (RNAi) and microarray analysis. We identified a list of *Lin28*-regulated genes. Among the genes on the list, we focused on neuropeptide Y5 receptor (*Npy5r*) and oocyte-specific homeobox 5 (*Obox5*) genes because of their relationship with ESCs based on previous reports and found the family members of *Npyr* and *Obox* genes also upregulated by *Lin28* downregulation in mESCs.

## Methods

### 1. Cell culture

J1 mESCs (SCRC-1010) were purchased from ATCC (Manassas, VA, USA). The cells were maintained in a feeder-free condition on 0.1% gelatin-coated plates. The culture medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco Invitrogen, Carlsbad, CA, USA), 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MI, USA), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 100 U/mL penicillin, 100 g/mL streptomycin, 2 mM glutamine (Gibco Invitrogen) and 1,000 U/mL leukemia inhibitory factor (Chemicon, Temecula, CA, USA).

### 2. siRNA transfection

*Lin28* siRNA (L-051530-01, Dharmacon, Denver, CO, USA) and control siRNA (D-001810-01-05, Dharmacon) were purchased. This siRNA was transfected into J1 cells with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Briefly, a total of  $5 \times 10^5$  cells were plated in 6-well plates and transfected using 200 pmol siRNA and 10  $\mu$ L of Lipofectamine 2000 per well. After 5 hours of incubation, 2 mL of growth medium was added to the cells. The next day, the medium was replaced with fresh growth medium. Following 24 hours of incubation, the cells were harvested for analysis.

### 3. Microarray analysis

Total RNA was extracted using TRIzol (Invitrogen) and biotinylated cRNA were prepared from 3  $\mu$ g of total RNA using an RNA amplification kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Following fragmentation, 12  $\mu$ g of cRNA was hybridized to the Affymatrix Genechip Mouse Genome 430 2.0 array (Affymatrix, Santa Clara, CA, USA) according to the manufacturer's instructions. The arrays were scanned by using a GeneChip scanner 3000 7 G (Affymatrix) and array data analysis was performed using GenPlex 3.0 software (Istech Co., Goyang, Korea).

### 4. RNA preparation and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol (Invitrogen) and 2  $\mu$ g of total RNA was used for the first cDNA synthesis using moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using the iQ SYBR Green Supermix PCR reagent (Bio-Rad, Hercules, CA, USA). Reactions were carried out using iCycler (Bio-Rad) and the results were evaluated with the iCycler real-time detection system software. For quantitation, the target genes were normalized against the glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene and performed by determining the cycle threshold value ( $C_T$ ). Relative quantitation of target gene expression was evaluated by the comparative  $C_T$  method. The PCR primers used in this study are listed in Table 1.

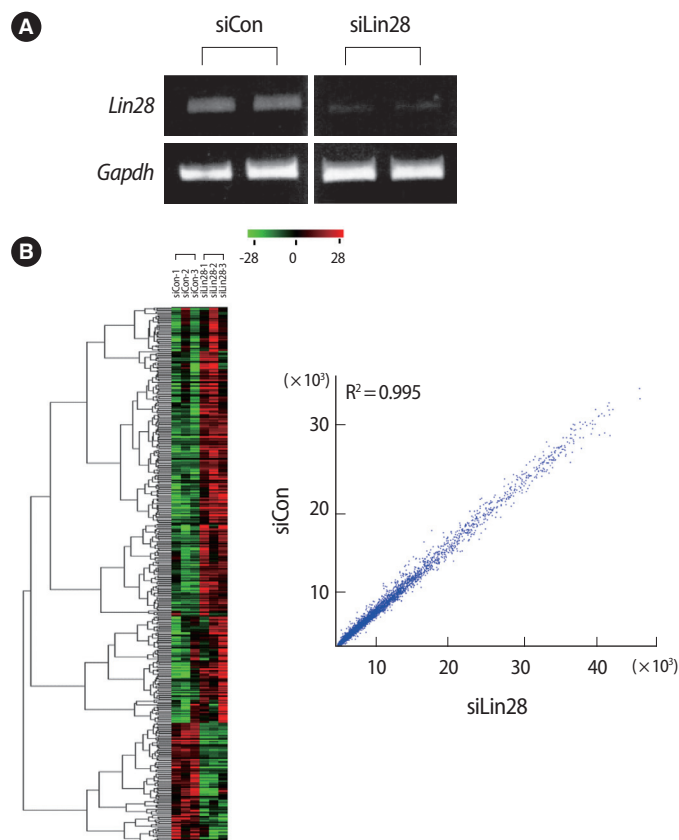
## Results

To determine the downstream effectors controlled by *Lin28* in mESCs, we first performed cellular knockdown of *Lin28* by RNAi. The control siRNA and *Lin28* siRNA were transfected into mESCs and the expression of *Lin28* was analyzed by RT-PCR. It was shown that the expression of *Lin28* was effectively suppressed compared to the control siRNA transfected cells (Figure 1A). Using the total RNA from three sets of independently prepared cells, we performed microarray analysis

**Table 1.** Reverse transcription-polymerase chain reaction primer sequences used in this study

Genes	Primer sequences	Amplicon (bp)
<i>Lin28</i>	F: 5'-GCGAAGATCCAAAGGAGACA-3' R: 5'-TGTGGATCTCTCTCTTCC-3'	206
<i>Mm.150234</i>	F: 5'-CACAGGAACAGACCCAAAG-3' R: 5'-CTCAGAGCTGTGCAGCAAAC-3'	146
<i>4930513N20Rik</i>	F: 5'-GCAGGGTCAGACTGCCTTAG-3' R: 5'-TACCCTGCTGGAGAGATGCT-3'	156
<i>Glins1</i>	F: 5'-GCACTCAGGTGGGAATATGG-3' R: 5'-GCACTCAGGTGGGAATATGG-3'	149
<i>Mm.226989</i>	F: 5'-AGGGCTCCTAATGCACACTG-3' R: 5'-CCAGTTATGCATGGAGCTT-3'	143
<i>Sntb1</i>	F: 5'-ACCTGAAAACAGGCAGCTA-3' R: 5'-CCTGATGCAGCAACCACAC-3'	152
<i>Ddx26b</i>	F: 5'-CTAAGCGAAGGCGGAGTATG-3' R: 5'-GGTGTCTGGATGCCAAAGG-3'	152
<i>Pfkfb2</i>	F: 5'-AGGCAGGCTGCTTTGTTAGA-3' R: 5'-CCATCACAGTGCATTCACC-3'	151
<i>Rnf14</i>	F: 5'-CTCAACTGTCCAGAGCCACA-3' R: 5'-ACGGGGACAATACCCACAT-3'	147
<i>Myst3</i>	F: 5'-CCAACCAGAACGAAGACCAT-3' R: 5'-GTCCTGCATGTTTGCAGCTA-3'	152
<i>Akap10</i>	F: 5'-AGCCTCAGTGTTCATCCTT-3' R: 5'-GATAGCGGAATGGTCTTGA-3'	152
<i>Zfp386</i>	F: 5'-GCCTTCTCTGACTGATTGGA-3' R: 5'-GAGCATTTACCAGGGAAGCA-3'	163
<i>Glb1l2</i>	F: 5'-GTGGATGGGCACCTTCTGTT-3' R: 5'-GTCAGCGAGGCTTTCATCTC-3'	147
<i>Phactr2</i>	F: 5'-GTGCACTGGCAAGCAAGATA-3' R: 5'-GCTTGGTCCGATCTGTTGT-3'	150
<i>Npy</i>	F: 5'-AGAGATCCAGCCCTGAGACA-3' R: 5'-GATGAGGGTGGAACTTGG-3'	147
<i>Npy1r</i>	F: 5'-ACAGCACTCTCCTCTGGTG-3' R: 5'-TGATTCCGTTGGTCTCACTG-3'	153
<i>Npy2r</i>	F: 5'-TCCCTGGTAATCCATGTGGT-3' R: 5'-TTCCACTCTCCATCAAGG-3'	147
<i>Npy4r</i>	F: 5'-TCAGCTAACCTGCCTTGGT-3' R: 5'-CCGTTCTGATTGCCCTGTT-3'	151
<i>Npy6r</i>	F: 5'-GTGGCTGGAACCCAGAGTA-3' R: 5'-CCTGGTGGGTGTAGATGTCA-3'	156
<i>Obox1/2</i>	F: 5'-CCTAACTGAATTTCAAACAATCGC-3' R: 5'-CCCAGAGGATGCTCACAATTCAG-3'	275
<i>Obox3</i>	F: 5'-TCCTGGTTCCATACCTGTTGT-3' R: 5'-GCAGGTATTCTTGGTATTCTTG-3'	179
<i>Obox4</i>	F: 5'-CCCTCATTGATCAACCCCTGG-3' R: 5'-AGTTTTGGTGCATACCTTGGAG-3'	240
<i>Obox5</i>	F: 5'-ATCAGACTGACATAGCAGTAG-3' R: 5'-ATGAAAGACTGAAAGGTGTC-3'	174
<i>Obox6</i>	F: 5'-CACAGCAAATGAGATCCAGAT-3' R: 5'-ATACCTGGCACTATCACAGGC-3'	274
<i>Gapdh</i>	F: 5'-ACCACAGTCCATGCCATCAC-3' R: 5'-TCCACCACCTGTTGCTGTA-3'	451

F, forward primer; R, reverse primer.

**Figure 1.** Microarray analysis of *Lin28* knocked down mouse embryonic stem cells (mESCs) by RNAi. (A) Control and *Lin28* siRNA were transfected into J1 mESCs. Suppressed expression of *Lin28* was shown after reverse transcription-polymerase chain reaction. Two microphotographs representative of the results are shown. *Gapdh* was used as a loading control. (B) Affymatrix GeneChip mouse genome 430 arrays were used to evaluate the genome-wide expression of *Lin28* knocked down mESCs. A heat map with hierarchical clustering (left panel) shows the results of the 500 selected genes after three independent microarray analyses. Scatter plots (right panel) comparing the expression pattern between the control and *Lin28* knocked down mESCs show the up- and down-regulated genes from the microarray. siCon, control siRNA transfected; siLin28, *Lin 28* siRNA transfected.

to compare the gene expression profiles of the control and *Lin28* knocked down mESCs. A heat map and scatter plot of the cDNA microarray depicts the high correlation between the control and experimental samples as well as the up- and down-regulated genes from microarray analysis (Figure 1B).

Among all of the genes, 500 genes were determined to be changed at least 2-fold in the *Lin28* knocked down mESCs (up-regulated, 384; down-regulated, 116). According to the Affymatrix microarray guideline, we applied MAS5 detection filtering for selection of differentially expressed gene. After the filtering procedure, we finally selected 31 genes (up-regulated, 24; down-regulated, 7). We omitted some genes

due to insufficient gene information and the rest of the genes (up-regulated, 19; down-regulated, 5) are listed in Table 2. Preliminarily, we examined the ordinary expression level of each selected gene in the mESCs (data not shown), and we performed RT-PCR analysis of the candidate genes using the cDNA from the control and *Lin28*-down-regulated mESCs to confirm the microarray results (Figure 2). We found that the expressions of several genes, *Mm.226989*, *Npy5r*, *Pfkfb2*, *Mm.150234*, *Zfp386*, and *Obox5* were significantly upregulated (Figure 2A), while *Phactr2* was downregulated (Figure 2B) in *Lin28* knocked down mESCs.

We focused on *Npyr* and *Obox* genes since their expression in ESCs was previously reported. *Npy*, one of the most abundant neuropeptides in the brain, exerts its functions by binding to six NPY receptors (Y1 to Y6) [18]. Mammals have five subtypes of NPY receptors, Y1 (*Npy1r*), Y2 (*Npy2r*), Y4 (*Npy4r*), Y5 (*Npy5r*), and Y6 (*Npy6r*). Thus, we examined the expression profiles of *Npy* and its receptors by quantitative real-time RT-PCR analysis using the cDNA of the control and *Lin28* knocked down mESCs (Figure 3). The results showed that *Npy2r* and *Npy5r* were significantly upregulated even though their basal expression levels were relatively low in mESCs. The results of other

receptors, *Npy1r*, *Npy4r*, and *Npy6r*, showed the tendency of increasing level of expression. However, the expression of *Npy* itself was slightly, but significantly downregulated in the *Lin28* knocked down mESCs.

The *Obox* family consists of 6 members and is exclusively expressed in the ovary and testes [19-21]. Kim et al. [22] reported that the *Obox* family is expressed in mESCs and *Obox4* regulates histone family gene expression. Thus, we examined the expression level of each *Obox* family gene in the *Lin28* knocked down mESCs in comparison with the control mESCs by quantitative real-time RT-PCR (Figure 4). The results showed that the expression levels of *Obox1/2*, *Obox3*, *Obox5*, and *Obox6* tended toward upregulation although the statistical significance value was low. Interestingly, however, the expression of *Obox4* was not much changed in the *Lin28* knocked down mESCs.

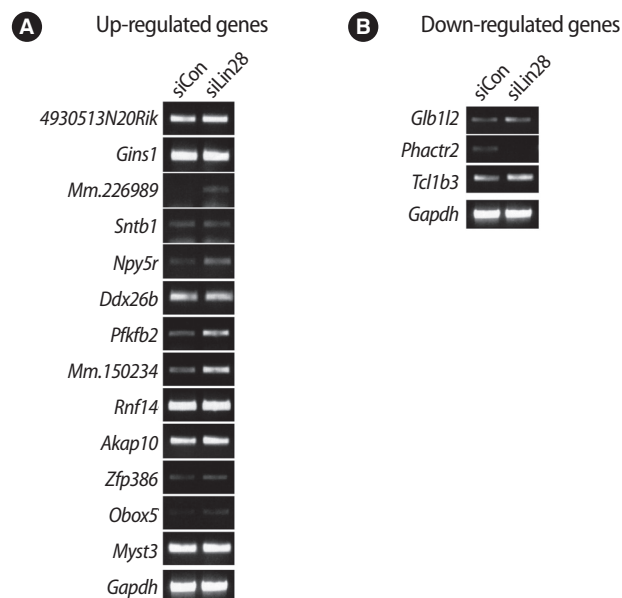
## Discussion

*Lin28* is spotlighted by its ability to promote iPS cell production from somatic cells. It is abundantly expressed in ESCs and plays crucial roles in proliferation and self-renewal of ESCs. A major role of *Lin28* in ESCs is to support the rapid growth of cells. However, the evidence used to

**Table 2.** List of the genes up- and down-regulated in mESCs after *Lin28* RNAi

Gene symbol	Gene title	Fold change
Upregulated genes		
<i>Mm.150234</i>	-	7.20
<i>Mm.226989</i>	-	6.97
<i>Sntb1</i>	Syntrophin, basic 1	4.15
<i>4833403115Rik</i>	4833403115Rik	3.91
<i>Rnf14</i>	Ring finger protein 14	3.14
<i>Akap10</i>	A kinase (protein kinase A, PRKA) anchor protein 10	2.82
<i>Obox5</i>	Oocyte specific homeobox 5	2.51
<i>Mm.404410</i>	-	2.49
<i>Npy5r</i>	Neuropeptide Y receptor Y5	2.36
<i>Igj</i>	Immunoglobulin joining chain	2.22
<i>Ddx26b</i>	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26B	2.21
<i>Pion</i>	Pigeon homolog (Drosophila)	2.20
<i>4930513N20Rik</i>	RIKEN cDNA 4930513N20 gene	2.12
<i>D3Bwg0562e</i>	DNA segment, Chr3, Brigham & Women's Genetics 0562 expressed	2.10
<i>Gins1</i>	GINS complex subunit 1 (Psf1 homolog)	2.05
<i>C76555</i>	Expressed sequence C76555	2.04
<i>Zfp386</i>	Zinc finger protein 386 (Kruppel-like)	2.04
<i>Myst3</i>	MYST histone acetyltransferase (monocytic leukemia) 3	2.03
<i>Pfkfb2</i>	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	2.00
Downregulated genes		
<i>Td1b3</i>	T-cell leukemia/lymphoma 1B, 3	0.49
<i>Gm2102</i>	Predicted gene 2102	0.48
<i>Phactr2</i>	Phosphatase and actin regulator 2	0.43
<i>Glb1l2</i>	Galactosidase, beta 1-like 2	0.32
<i>Fam55b</i>	Family with sequence similarity 55, member B	0.09

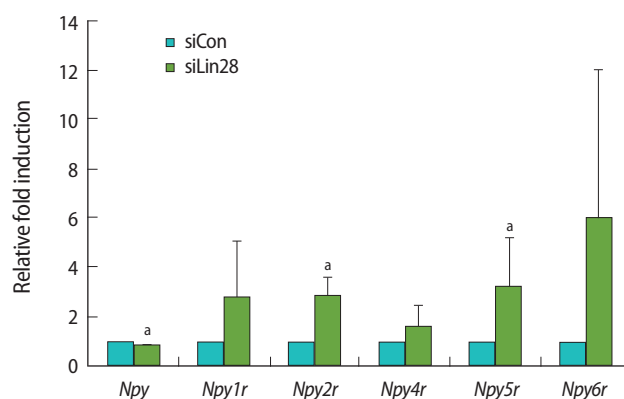
mESCs, mouse embryonic stem cells; RNAi, RNA interference.



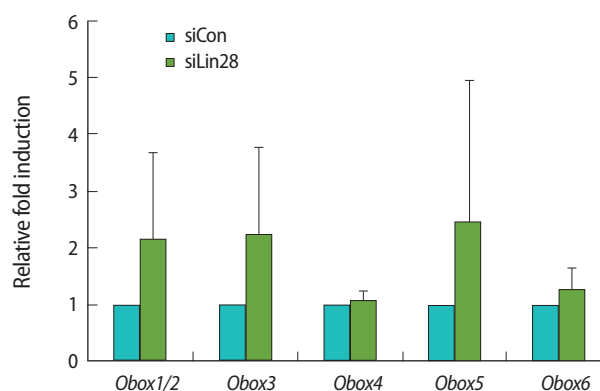
**Figure 2.** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the selected genes after microarray analysis of the *Lin28* knocked down mouse embryonic stem cells (mESCs). The expression of target genes selected by statistical method and filtering procedure after microarray analysis was examined by RT-PCR using specific primers with cDNA from control and *Lin28* knocked down mESCs. Microphotographs representative of the results of (A) up-regulated and (B) down-regulated genes after RT-PCR are shown. *Gapdh* was used as a loading control. siCon, control siRNA transfected; siLin28, *Lin28* siRNA transfected.

explain the role of *Lin28* in ESCs is controversial. Several reports have suggested that *Lin28* facilitates cell proliferation by binding a subset of mRNA involved in the cell cycle and metabolism [11,12,23]. However, Darr and Benvenisty [10] reported that downregulation of *Lin28* did not lead to differentiation, but rather *Lin28* overexpression impaired the ability of cells to grow at clonal densities. Also, like other reprogramming factors, *Lin28* has been reported to play a role in controlling the aggressiveness, recurrence, and metastasis of cancers [24-27]. Therefore, it is necessary to confine the corresponding downstream effectors of *Lin28* in various cell types, including stem cells. In this study, we performed microarray analysis using the total RNA from transiently *Lin28* knocked down mESCs by RNAi, and found that *Npyr* and *Obox* family members showed a tendency toward upregulation by *Lin28* RNAi (Figures 3, 4).

The growth factor properties of NPY were demonstrated in various cell types including neurons, endothelial cells, vascular smooth muscle cells, and adipocyte precursor cells [28-31]. In the case of ESCs, it has been reported that NPY and its receptors are involved in maintaining the self-renewal and proliferation of hESCs [32]. NPY supports the long-term growth of undifferentiated hESCs in the feeder-free condition and both Y1 and Y5 receptors are involved in the NPY-me-



**Figure 3.** Upregulation of the *Npyr* family in *Lin28* knocked down mouse embryonic stem cells (mESCs). Relative expression of *Npy* and *Npyr* in *Lin28* knocked down mESCs was confirmed by quantitative real-time reverse transcription-polymerase chain reaction. The expression levels were calculated by computed tomography values, and the relative fold change was determined to compare the values of control mESCs. The data are represented as the mean  $\pm$  SD from 3 independent experiments. The alphabet a represents statistical significance at  $p < 0.05$ . siCon, control siRNA transfected; siLin28, *Lin28* siRNA transfected.



**Figure 4.** Upregulation of the *Obox* family in *Lin28* knocked down mESCs. Relative expression of the *Obox* family in *Lin28* knocked down mESCs was confirmed by quantitative real-time reverse transcription-polymerase chain reaction. The expression levels were calculated by computed tomography values, and the relative fold change was determined to compare the values of the control mESCs. The data are represented as the mean  $\pm$  SD from 3 independent experiments. *Obox*, oocyte-specific homeobox; mESCs, embryonic stem cells; siCon, control siRNA transfected; siLin28, *Lin28* siRNA transfected.

diated activation. *Npy1r* plays an important role in the regulation of mesenchymal progenitor cell differentiation [33]. Through the Y1 receptor, NPY directly inhibits the differentiation of mesenchymal progenitor cells. Therefore, since *Npy* and its receptor family are expressed in ESCs, it would be possible that *Lin28* regulates the *Npyr* family to control the proliferation of mESCs. We could not, however, rule out

the possibility that the upregulation of the Npyr family by *Lin28* RNAi is through indirect regulation.

The Obox gene family has been known to be exclusively expressed in germ cells. Given the many similarities between germ cells and ESCs, sets of genes may express in both germ cells and ESCs. The expression of the Obox family is detected in mESCs as well as in oocytes [21,22]. Basically, *Obox4* has been known to regulate the cAMP-dependent signaling cascades that maintain the intact germinal vesicle membrane of oocytes [21]. It has been suggested that *Obox4* plays the following role in mESCs: *Obox4* expression leads mESCs into a differentiated state with decreased expression levels of *Oct4* and *Sox2* [22]. This report also suggested that *Obox4* might have a different role in mESCs than do other Obox family members such as *Obox6*. Interestingly, *Obox4* is expressed as an unspliced transcript in mESCs, and the unspliced intron is spliced out upon the differentiation of mESCs [22]. Therefore, it is presumed that Obox family members other than *Obox4* have a regulatory role in undifferentiated mESCs. In this study, we found that the Obox gene family, other than *Obox4*, had a tendency toward upregulation in mESCs by *Lin28* RNAi. Thus, we propose that *Lin28* might regulate the expression of the Obox family genes selectively to control the differentiation of mESCs in a fine-tuned manner.

*Lin28* has been known to be an RNA-binding protein and function as a post-transcriptional regulator [9-12]. Our results showed that *Lin28* also regulated the downstream effectors, Npyr and Obox family genes, functioning in the control of cell destination at the transcriptional level. Until now, it has not been clearly revealed how the expression of the genes, such as Npyr and Obox families, in ESCs is regulated. Thus, our results could also provide an important clue for the understanding of the regulation mechanism of gene expression in ESCs. Further elucidation of the other target genes of *Lin28* in ESCs is needed for understanding the multiple functions of *Lin28* in ESCs.

### Conflict of interest

No potential conflict of interest relevant to this article was reported.

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