博士論文

The Role of Importin-βs in the Maintenance and Lineage Commitment of Mouse Embryonic Stem Cells

マウス胚性幹細胞の維持、および系譜特異的 な細胞分化におけるインポーチン-β の役割

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ABSTRACT

The Importin-β family is composed of proteins that recognize nuclear localization signals (NLS) and nuclear export signals (NES). These proteins play important roles in various nucleocytoplasmic transport processes in cells. Here, I examined the expression patterns of 21 identified Importin-β genes in mouse embryonic stem cells (mESCs), mouse embryonic fibroblast (MEF) and mESCs differentiated into neural ectoderm (NE) or mesoendoderm (ME), and I observed a striking difference in the Importin-β mRNA expression levels among them. I also found that knockdown of select Importin-β genes led to suppression of Nanog, and altered the balance of Oct4/Sox2 expression ratio, which is important for NE/ME lineage choice. Furthermore, I demonstrated that knockdown of *XPO4, RanBP17, RanBP16,* or *IPO7* differentially affected the lineage selection of differentiating mESCs. More specifically, knockdown of *XPO4* selectively stimulated the mESC differentiation towards definitive endoderm, while concomitantly inhibiting NE differentiation. *RanBP17* knockdown also promoted endodermal differentiation with no effect on NE differentiation. *RanBP16* knockdown caused differentiation into ME, while *IPO7* knockdown inhibited NE differentiation, without obvious effects on the other lineages. In addition, I also found that *RanBP17*or *IPO7* has limited potential in cellular reprogramming of MEFs to mouse induced pluripotent stem cells (miPS cells). Collectively, my results suggest that Importin-βs play important roles in cell fate determination processes of mESCs, such as in the maintenance of pluripotency or selection of lineage during differentiation.

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BACKGROUND OF THE STUDY

Nucleocytoplasmic transport system and Importins

The eukaryotic cell is defined by a set of complex subcellular endomembrane compartmentalization that gives rise to various intricate trafficking pathways. The nucleocytoplasmic transport system is an important system, which regulates the flow of various substrates between the cytoplasm and the nucleus. Central to this transport system are soluble proteins collectively known as Importins (α and β), which mediate most of the nucleocytoplasmic pathways in the cell. Previous works from our laboratory contributed to the better understanding of Importin-α. The study of Sekimoto *et* al. (1997) [1] revealed the existence of different subtypes of Importin-α in eukaryotic cell, which paved way to the discovery and analysis of different types of nuclear localization signal (NLS) that can be recognized by these different subtypes during protein cargo transport. Another noteworthy mentioning is the study of Yasuhara *et* al. (2007) [2], which demonstrated the occurrence of Importin-α subtype switching during neural differentiation in embryonic stem cells. This study highlighted the importance of coordinated regulation in Importin-α subtypes and their transcription factor cargoes. This study also indicated the possible role of nuclear transport factors as key coordinators in cell-fate determination. Like the Importin-α, the Importin-β family is another equally important player in the nucleocytoplasmic transport system. However, there are limited reports on these proteins. I believe, like Importin-α, the Importin-β proteins are also associated with various cellular events like maintenance of pluripotency or lineage selection in embryonic stem cells. Thus, my study focused on Impotin-β family with the aim of contributing some information on their functional role, particularly in mouse embryonic stem cells (mESCs).

Importin-β family

The Importin-β family is composed of proteins that mediate the majority of macromolecular transport between the nucleus and the cytoplasm. Specifically, proteins belonging to this family are involved in the shuttling of cargo proteins and some RNAs across the nuclear pore complex (NPC). Importin-β proteins accomplish either nuclear import and are called importins or nuclear export and are called exportins. Only some of them take a part in both import and export processes. This nucleocytoplasmic transport function is mediated by their recognition of either nuclear localization signal (NLS) for the nuclear import of cargo proteins or nuclear export signal (NES) for the nuclear export of cargo proteins [3,4,5]. This signal (receptor)-mediated Importin-β and protein cargo interaction is regulated by a member of Ras superfamily, Ran (RanGTP).

Nuclear transport model

A simple nuclear transport model is shown in Fig. 1, which summarizes the role of Importin-βs during the import/export of cargo proteins between the nucleus and the cytoplasm. For nuclear import, cargo proteins can be transported from the cytoplasm into the nucleus in two ways. First, the NLS of a cargo protein is recognized by the heterodimeric Importin complex, composed of Importin-α and Importin-β1. The Importin-α subunit binds to NLS, whereas Importin-β1 subunit, which also possesses Ran binding activity, mediates the docking at the NPC to the nucleoporins. Together, the cargo protein and the Importins (α and β1) form a ternary complex. The translocation of this ternary complex from the cytoplasm to the nucleoplasm is by the weak hydrophobic interactions of Importin-β1 with the nucleoporins of the NPC. During translocation, nuclear RanGTP binds to the

Importin-β1 subunit, which leads to the dissociation of the Importin heterodimer that cause the final delivery of proteinNLS and Importin-α to the nucleoplasm, while the Importin-β1-RanGTP returns to the cytoplasm. Second, the NLS of the cargo protein is directly recognized by Importin, forms a complex and immediately transported into the nucleus via the weak hydrophobic interactions of importin with the nucleoporins of the NPC. In the same way, the binding of RanGTP to the Importin causes the dissociation of the cargo protein, while the Importin-RanGTP complex is shuttled back to the cytoplasm. For the nuclear export, the NES of cargo protein forms a complex with Exportin (example Exportin 1) and RanGTP. This complex passes through the nucleoporins of the NPC towards the cytoplasm. In the cytoplasm, the hydrolysis of RanGTP to RanGDP triggers the dissociation of the complex into free proteinNES, Exportin and RanGDP. [3,6,7].

As mentioned earlier, a small GTPase protein, Ran regulates most Importin-β and protein cargo interactions. GTP bound Ran (RanGTP) is highly concentrated in the nucleus than in the cytoplasm due to the presence of Ran's guanine-exchange factor RCC1, which is located in the chromatin. In the cytoplasm, majority of Ran is bound to GDP (RanGDP) due to the presence of a GTPase activating protein RanGAP1 that hydrolyzes RanGTP to RanGDP. The asymmetric distribution of RCC1 and RanGAP1 across the nuclear envelope creates a RanGTP/GDP gradient, which directs the nucleocytoplasmic transport of proteins [5].

The earlier described nucleocytoplasmic transport system is very important since proteins are translated in the cytoplasm but many have functional roles in the nucleus. Therefore, the Importin-βs as transporters are involved in many essential cellular processes, which make these proteins biologically important [5]. Though,

they also have other non-transport functional roles that are equally important to cellular viability [8].

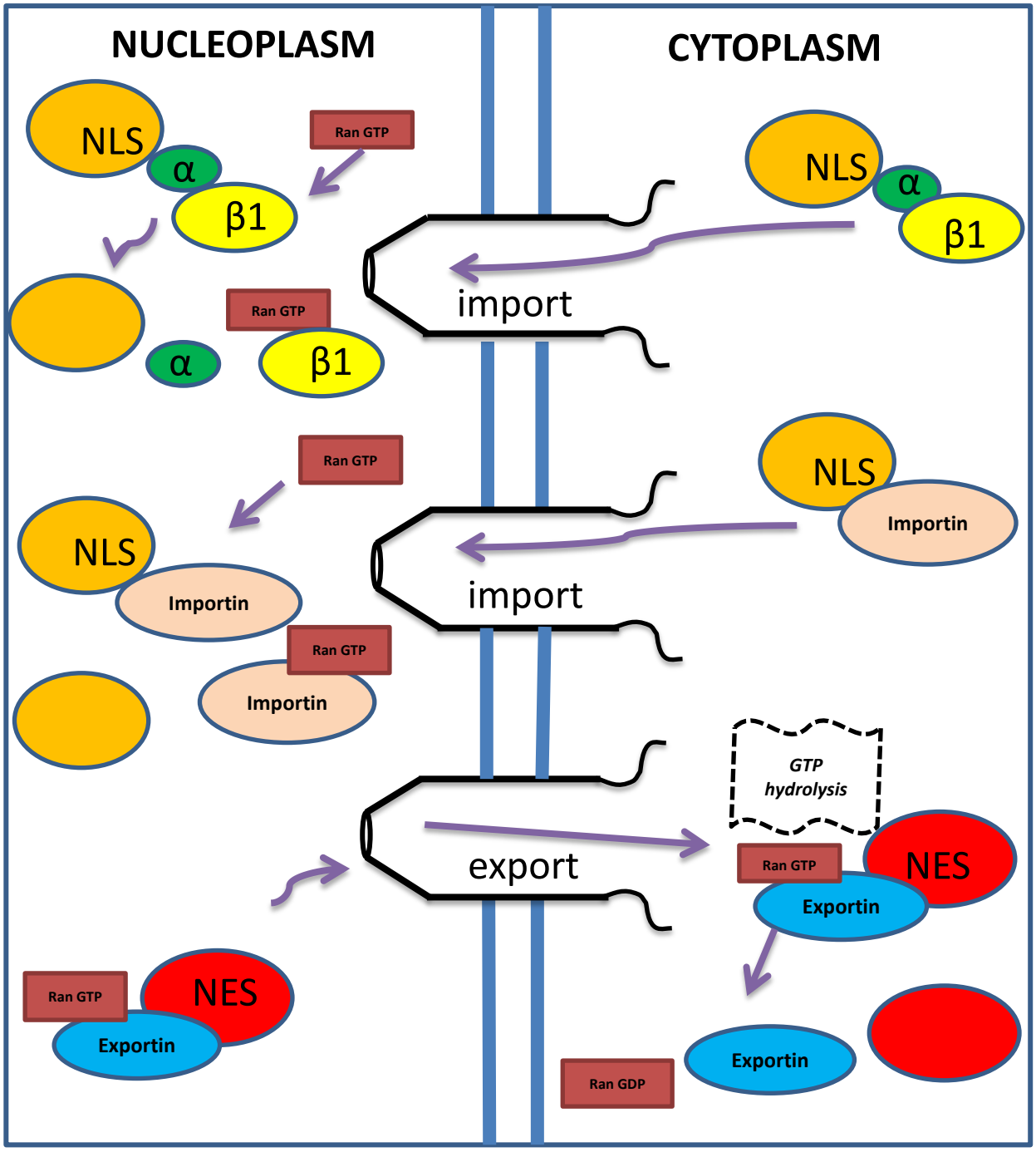


Figure 1. Nucleocytoplasmic transport mechanism model.

Mouse embryonic stem cells (mESCs)

More than 20 proteins in mouse were identified under the Importin-β family. The names of these proteins are shown in Table 1. However, few reports on Importin-βs are available in mouse, most especially in mESCs, considering that they play varied functional roles in the cell. Thus, I conducted this study to further understand the role of Importin-βs in the cellular events of mESCs.

The derivation of mESCs was first reported in 1981 [9]. These cells were originally derived from the inner cell mass (ICM) or epiblast of mouse embryos. They exhibit an almost unlimited proliferative capacity in culture and maintain their pluripotent potential to differentiate into all cell lineages in the body. Mouse embryonic stem cells are commonly maintained in serum-containing medium with the addition of a cytokine, leukemia inhibitory factor (LIF) to allow them to proliferate in the absence of feeder cells. LIF modulates mouse ES cells through the activation of STAT3 (signal transducers and activators of transcription) protein, which plays a central role in the maintenance of the pluripotential stem cell phenotype [10]. The proliferative and developmental potential of mESCs ensures an unlimited supply of specific cell types for research, which makes the mESC a good cell candidate for this study.

mESC differentiation and cellular reprogramming studies

My research also focused on the role of Importin-βs in the early differentiation of mESCs and in their potential use in the cellular reprogramming of mouse embryonic fibroblasts (MEFs) to mouse induced pluripotent stem cells (miPS cells). The

Table 1. Importin-β proteins identified in mouse.

Importin-β Protein	Other Nomenclature (Synonym)
TRN1	AU021749; D13Ertd688e; IPO2; Kpnb2; MIP;
TRN2	1110034O24Rik; AA414969; AI464345; AI852433; IPO3; Knpb2b; Kpnb2b; Tnpo2
TRN3	5730544L10Rik; C430013M08Rik; C81142;D6Ertd313e;mKIAA4133; Trn-SR
IPO4	8430408O15Rik; AA409693; Imp4a; RanBP4
IPO5	1110011C18Rik; 5730478E03Rik; AA409333; C76941; IMB3; Kpnb3; Ranbp5
IPO7	A330055O14Rik; C330016G14; Imp7; Ranbp7
IPO8	6230418K12Rik; Abcc10; C130009K11Rik; MRP7; OM-1; Om1; Ranbp8
IPO11	1700081H05Rik; 2510001A17Rik; AI314624; AW555235; E330021B14Rik; Ranbp11
IPO13	Imp13; Kap13; Ranbp13
XPO-t	1110004L07Rik; 3110065H13Rik; AI452076; C79645; EXPORTIN-T
XPO1	AA420417; Crm1; Exp1
XPO4	B430309A01Rik; mKIAA1721
XPO5	2410004H11Rik; 2700038C24Rik; AI648907; AW549301; Exp5; mKIAA1291; RanBp21
XPO6	2610005L19Rik; AI854665; AL022631; C230091E20Rik; mKIAA0370; R75304; Ranbp20
Ran BP6	C630001B19
Ran BP9	IBAP-1; Ibap1; RanBPM
Ran BP10	4432417N03Rik
Ran BP16	Xpo7; 4930506C02Rik; BB164534; mKIAA0745
Ran BP17	4932704E15Rik
Cse1L	2610100P18Rik; AA407533; Capts; Cas; Xpo2
KPNB1	AA409963; Impnb; IPOB , Importinβ1

differentiation of mESCs also provides model systems to study early developmental events in mammals. Through *In vitro* manipulation of mESCs, their differentiation can be directed towards a specific lineage which ultimately results to a specific cell type. On the same note, cellular reprogramming promises unlimited research and medical applications. Cellular reprogramming is the process of converting one cell type to another. By reverting a specific cell like the MEF to its pluripotent condition (miPS cell), this reprogrammed cell can be directed to become another cell type.

Highlights of the study

This study is the first report on the expression patterns of Importin-β genes in mESCs, mouse embryonic fibroblasts (MEFs) and in mESCs differentiated into neural ectoderm (NE) or mesoendoderm (ME). My study also showed the involvement of Importin-βs in the cellular events of mESCs like in the maintenance of pluripotency and in their differentiation to a specific lineage. Specifically, my findings showed that appropriate expression patterns of Importin-β proteins in mESCs are important in the maintenance of pluripotency and lineage choice during differentiation. This study also showed the limited application of some Importin-βs in cellular reprogramming of MEFs to mouse induced pluripotent stem cells (miPS cells).

1. INTRODUCTION

The importin-β family, comprising importins and exportins, is a group of proteins of molecular weights ranging from 90 to150 kDa. Proteins belonging to this family have low sequence identity (10-20%) and all contain helical HEAT repeats [3,4,5]. These proteins recognize nuclear localization and export signals (NLS/NES, respectively), bind weakly to phenylalanine-glycine (FG)-repeats in the nuclear pore complex (NPC), and play roles in the nucleocytoplasmic transport processes of various proteins [11,12]. Importin-β-cargo interactions are regulated by the small GTPase , Ran [7]. Because the number of Importin-βs is limited, each member protein mediates the transport of multiple protein cargoes [13]; thus, Importin-βs are essential for diverse cellular processes such as gene expression, signal transduction, and oncogenesis [5]. Moreover, they are involved in non-transport processes such as mitosis, centrosomal duplication, and nuclear envelope assembly [8].

The Importin-β family comprises at least 20 proteins in humans and 14 in *S. cerevisiae* [8,13]. Approximately 11 of these proteins in humans and 10 in *S. cerevisiae* are reported to mediate nuclear import through recognition of NLS [5]. However, limited data are available for mouse models, particularly with regard to embryonic stem cells (mESCs).

Thus, this study was conducted to understand the roles of Importin-βs in the different cellular events of mESCs and their potential use for cellular reprogramming. To my knowledge, this is the first study on the genetic expression patterns of the Importin-β family in mESCs and their differentiated germ layer cells. My results reveal a possible association between the expression of some Importin-βs and the

maintenance of pluripotency or lineage selection during the differentiation of mESCs. In this study, I also found that some Importin-βs have limited application in cellular reprogramming.

2. MATERIALS AND METHODS

2.1. Culture of mouse embryonic stem cells

Feeder-free mouse embryonic stem cells (EB3) [14] were used for all experiments. EB3 cells were maintained on 0.1% gelatin-coated surfaces in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM of MEM nonessential amino acid (GIBCO), 100 mM of MEM sodium pyruvate (GIBCO), 0.1 mM β-mercaptoethanol (Sigma Chemical) and LIF at 37°C in 5% CO_2. Cells were passaged every 2 or 3 days.

2.2. Culture of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) isolated from C57BL/6NCrSlc (SLC) were cultured in DMEM supplemented with 10% FBS, at 37°C in 5% CO_2.

2.3. Mouse embryonic stem cell differentiation

For differentiation toward neural ectoderm (NE) or mesoendoderm (ME) lineages, 2 × 10^6 feeder-free EB3 cells were plated and incubated for 48 h on a 0.1% gelatin-coated surface of 100 mm culture dish with serum-free N2B27 media without LIF [15,16]. This was followed by the addition of 500 nM retinoic acid (RA) for NE differentiation [17] or 3 μM CHIR99021 for ME differentiation [18]. Treated cells were

incubated for an additional 48 h before they were trypsinized and collected for quantitative PCR analysis.

2.4. RNA extraction and reverse transcription

For all cells, RNA was extracted with TRIZOL (Invitrogen), DNase treated (Zymo Research), and reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche). All procedures were performed according to the manufacturer's recommendations. The reverse transcription was performed at 25°C for 10 min, 50°C for 60 min, and 85°C for 5 min.

2.5. Reverse transcription PCR and quantitative PCR

Reverse transcription (RT) PCR was conducted using the initial step discussed in 2.4. Following cDNA synthesis, a 40 ng template for each of the test samples was amplified in GeneAmp™ PCR System 9700 (Applied Biosystems) using KOD Plus (Invitrogen), according to the manufacturer's recommendation. The PCR conditions were set at a pre-denaturation temperature of 94°C for 2 min, 35 cycles of denaturation temperature at 94°C for 15 s, annealing temperature at 55°C for 30 s (for Brachyury and Actin) or 60°C for 30 s (for Sox1), and extension at 68°C for 30 s. This was followed by a final extension temperature of 72°C for 5 min.

All Quantitative (Q) PCR analysis was performed on a 384-well plate with an ABI PRISM 7900HT system (Applied Biosystems) using FastStart Universal SYBR Green Master [Rox] (Roche). The qPCR reaction consisted of a holding temperature of 95°C for 30 s, and 40 cycles of 95°C for 15 s, 60°C for 30 s, and a standard dissociation stage. Standard curves were generated for all target genes with serial dilutions of total RNA from EB3 cells at 0.8, 4, 20, and 100 ng. Total RNA from

experimental cells was diluted to 20 ng and used as a template. The relative target mRNA expression levels were determined using the Pfaffl method and all values were normalized using GAPDH mRNA levels.

2.6. siRNA-oligonucleotide treatment

For all transfections, 2×10^5 feeder-free EB3 cells were seeded onto 0.1% gelatin-coated surfaces of 6-well plates with 2 mL of Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM of MEM nonessential amino acid (GIBCO), 100 mM of MEM sodium pyruvate (GIBCO), 0.1 mM β-mercaptoethanol (Sigma), and LIF . Immediately after plating, the cells were transfected with 2 different siRNA constructs for each of the target genes (i.e., *RanBP17, XPO4, IPO7, KPNB1, Cse1L* and *RanBP16*) using Lipofectamine™ RNAiMAX (Invitrogen), and incubated at 37°C, in 5% CO_2. After 48 h incubation, the medium was changed using 2 mL of fresh medium without LIF, and another transfection was performed according to the same procedure. The cells were incubated for an additional 48 h before they were trypsinized and collected.

2.7. Induced differentiation in siRNA-oligonucleotide treated EB3 cells

All transfections were carried out using the method described in 2.6. After the initial 48 h incubation in enriched DMEM with LIF, siRNA-oligonucleotide treated EB3 cells were induced to differentiate toward either NE or ME with the addition of 500 nM retinoic acid (RA) or 3 μM CHIR99021, respectively. The RA- or CHIR99021-treated cells were maintained in LIF-withdrawn enriched DMEM for another 48 h before they were trypsinized and collected for quantitative PCR analysis.

2.8. Plasmids for cellular reprogramming

Retroviral vectors (pMXs) expressing Oct4, Sox2, Klf4 and c-Myc (obtained from Addgene) were used for this study. Two highly expressed Importin-βs in mESCs, specifically *RanBP17*and *IPO7,* were considered for overexpression. Each Importin-β gene was PCR amplified (primer sequences are shown in Supplementary Table 1). The pMXs-RanBP17 or pMXs-IPO7 was created by inserting the coding sequence of *RanBP17* or *IPO7* into the BamHI and SalI sites of the multi-cloning site of the pMXs-Flag vector (obtained from Addgene), which was used as template. Then the newly constructed plasmids were cloned and sequenced.

2.9. Induction of Cellular Reprograming

Mouse- induced pluripotent stem cells (miPS cells) were generated following the method previously described [19] using pMXs retroviruses expressing mouse Oct4, Sox2, Klf4 and c-Myc (Addgene) together with *RanBP17* or *IPO7*. Plat-E cells were transfected with the pMXs retroviral vectors using Fugene HD transfection reagent (Roche). The medium was changed 24 h after transfection and supernatant were collected and filtered through 0.45- µm filter after 48 h. Filtered supernatant containing viruses were used to infect mouse embryonic fibroblasts (MEFs). After 24 h post-infection, 1×10^4 cells were re-plated onto a gelatin-coated surface 100-mm dish with mitomycin C-treated feeder cells. The culture medium was replaced with ES medium containing 20% knockout serum replacement (Invitrogen) 24 h after infected MEFs re-plating. The medium was changed every other day. Alkaline phosphatase staining was performed using the leukocyte alkaline phosphatase kit (Sigma).

2.10. Western Blot

Cells were lysed with RIPA buffer (50 mM Tris-HCl at pH 8.0, 0.15 M NaCl, 1 mM EDTA at pH 8.0, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 6 mM NaF, 1 mM Na_3VO_4, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 1 µg/mL pepstatin) and incubated for 15 min on ice. The samples were centrifuged at 1500 rpm for 10 min at 4°C. Supernatants from samples were collected and total protein concentrations were determined using a BCA protein assay kit (Pierce). Then 20 µg of each protein sample was separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 3% skim-milk in TBST buffer (50 mM Tris-HCl (pH 8.0), 100 nM NaCl, and 0.1% Tween 20) for 30 min at room temperature, the membrane was incubated overnight at 4°C with primary antibodies anti- mouse Oct3/4 (BD Transduction Laboratory), anti- mouse Nanog (ReproCELL), or anti- mouse Sox2 (EMD Millipore) as suggested by the manufacturer. After incubation for 45 min with secondary antibodies conjugated to horseradish peroxidase, bands were visualized using Pierce western blotting substrate (Thermo Scientific). All protein levels were normalized to GAPDH levels (Ambion).

2.11. Nucleotide sequences

All oligonucleotide sequences used for this study are summarized in Supplementary Table 1.

2.12. Statistical analysis

Statistical analysis was carried out using the unpaired Student's *t* test. The *p* values ≤ 0.05 indicated a statistically significant difference, while *p values* ≤ 0.01 indicated a highly significant difference (*, $p < 0.05$; **, $p < 0.001$).

3. RESULTS

3.1. Importin-β expression levels in mESCs vs. MEFs

I identified 21 mouse Importin-β genes in the database, designed primers and optimized conditions for qPCR analysis (Primer sequences are shown in Supplementary Table 1). The mESCs, particularly EB3 cells, were maintained in the pluripotent state by using LIF and enriched DMEM with methods previously described. I also cultured mouse embryonic fibroblasts (MEFs) using the methods described earlier. Then I compared the relative expression levels of Importin-βs in mESCs and MEFs by qPCR from 3 independent experiments (Fig. 2). Interestingly, the *RanBP17* mRNA expression level was found to be much higher in mESCs than in MEFs. I also found that other Importin-βs such as *IPO7, IPO11, XPO1, XPO4,* and *Cse1L* were also more highly expressed in mESCs compared to MEFs. This result is similar to previous reports of highly expressed Importin-β genes in different pluripotent cells such as hESCs, rat iPS cells, human iPS cells, and mESCs using microarray analysis [20,21,22]. On the other hand, I also observed that the *IPO4* mRNA expression level was much lower in mESCs than in MEFs. Importin-βs *IPO13, RanBP10, XPOt, TRN2, RanBP6, XPO5,* and *TRN1* were expressed at lower levels in mESCs than in MEFs.

Interestingly, the closely-related *RanBP16* (also designated *XPO7*) and *RanBP17,* which reportedly share 67% amino acid sequence identity [23, 24], exhibited different expression levels; that is, the *RanBP16* mRNA expression levels were similar in both mESCs and MEFs, while *RanBP17* was highly expressed in mESCs, but not in MEFs. This suggests that *RanBP16* and *RanBP17* may vary in function in mESCs,

which is consistent with results from a previous study reporting different activities of these proteins in other cells [25]

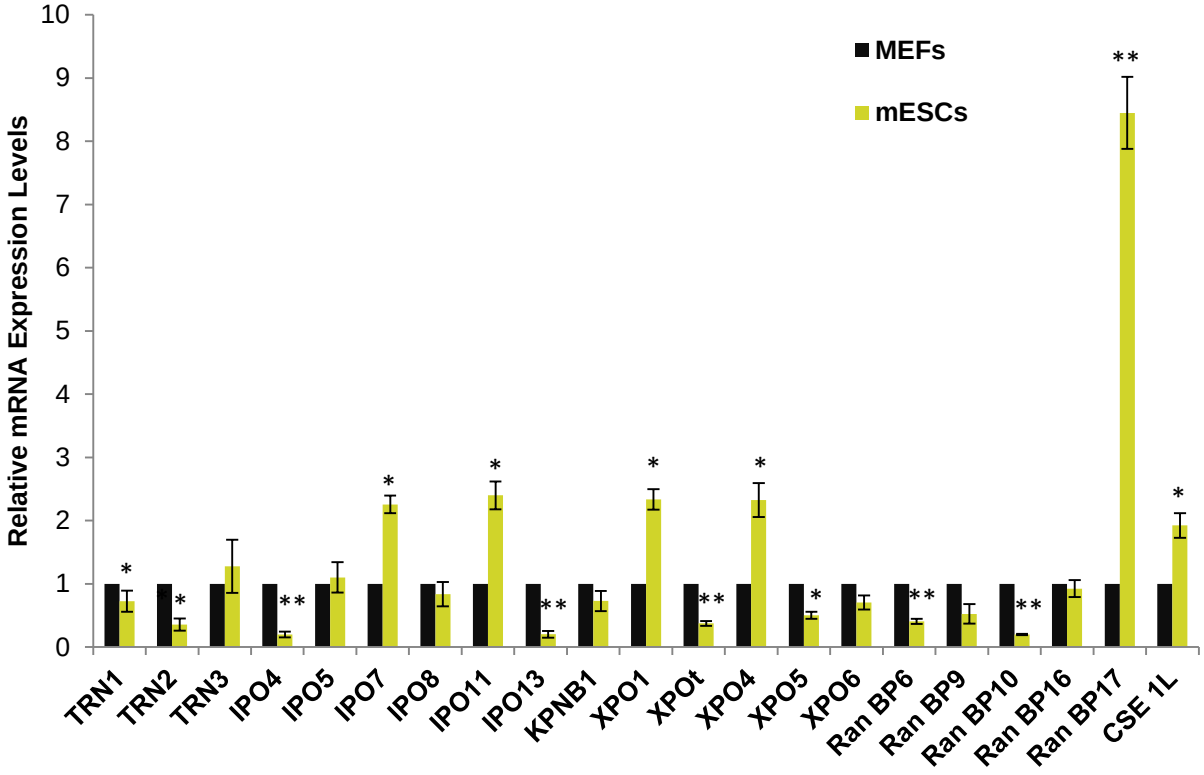


Figure 2. Importin-βs in mESCs. Expression of Importin-βs in mouse embryonic stem cells (mESCs) was assessed by QPCR and the changes are presented as a fold change relative to their expression levels in mouse embryonic fibroblasts (MEFs) used as controls. Importin-βs mRNA levels were normalized to GAPDH levels. Significance was assessed and compared with the levels in the control using unpaired Student's *t* test (*$p < 0.05$; **$p < 0.01$). Error bars represent SEM from 3 independent experiments.

3.2. Importin-β expression levels in cells differentiated into germ layer progenitors from mESCs In vitro

I also determined the relative expression levels of Importin-β genes in cells differentiated from mESCs to germ layer progenitors, namely, cells of the neural ectoderm (NE) and mesoendoderm (ME). I propagated EB3 cells maintained in a

pluripotent state by using LIF in enriched DMEM; transferred them to N2B27, a defined medium without differentiation signals [15,16]; and incubated them for 48 h as previously described. The 48 h “temporal window” is needed for cells removed from pluripotency-promoting conditions to respond to either NE- or ME-inducing signals as reported by Jackson *et al.* [26]. Then I induced the cells to transform into either NE or ME cells using retinoic acid (RA) or CHIR99021, respectively.

After 48 h in N2B27 supplemented with RA, the cells exhibited signs of NE differentiation and subsequently triggered the activation of the NE marker, Sox1 (Fig. 3A and Fig. 3B). This result was consistent with those of published studies reporting Sox1 activation following RA addition [17,27,28]. Likewise, cells responded to CHIR99021 and differentiated into ME with the activation of the core mesodermal regulator Brachyury (Figs. 3A-3B), as reported previously [29].

A comparison of the mRNA expression levels of Importin-βs in progenitor germ layer cells and mESCs (Figs. 3C-3D) revealed a striking difference between levels in NE and ME, and levels in mESCs (Fig. 2). In NE cells, we observed that *IPO13* was the most highly expressed gene, although *RanBP10, RanBP9, RanBP6, IPO4, XPOt, RanBP16, XPO5,* and *XPO6* were also highly expressed. On the other hand, the other Importin-β members in this study were expressed in NE at levels comparable to the levels in mESCs. In ME cells, *RanBP6* was the most abundantly expressed Importin-β gene, while *IPO11* was found to be expressed at the lowest level. *IPO13, IPO4, XPO6,* and *RanBP9* were also readily detected, whereas the remaining Importin-βs were expressed at levels similar in mESCs.

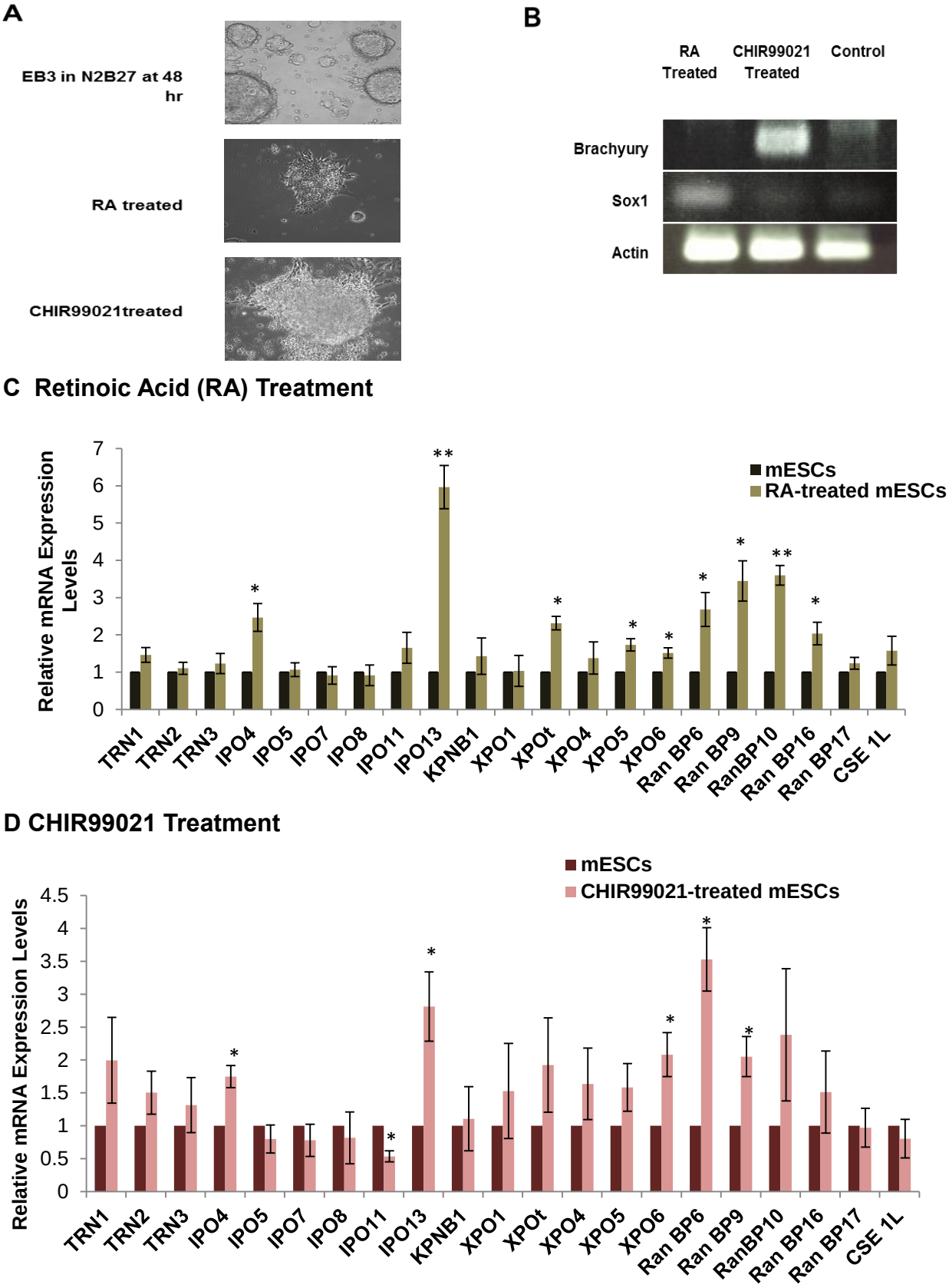


Figure 3. Importin-β mRNA expression levels of mESCs differentiate into germ layer progenitors *in vitro*. (A) Phase-contrast images of EB3 cells exhibiting signs of differentiation following retinoic acid (RA) or CHIR99021 treatment. (B) RT-PCR expression analysis of early lineage markers Brachyury (for mesoderm) and Sox1 (for neural

ectoderm). (C,D) Expression of Importin- βs in RA- or CHIR99021-treated mESCs was assessed by QPCR and is presented as a fold change relative to their expression levels in non-treated mESCs used as controls. Importin-βs mRNA levels were normalized to GAPDH levels. Significance was assessed and compared with the control using unpaired Student's *t* test (*$p < 0.05$; **$p < 0.01$). Error bars represent SEM from 3 experiments.

3.3. Effect of knockdown of Importin-βs on the expression of Nanog

Given the variation in Importin-β gene expression patterns in mESCs, MEF, NE cells, and ME cells, I considered whether this might have a functional impact on either the maintenance of pluripotency or lineage selection during differentiation. To address this, I selected and knocked down Importin-β genes that were highly expressed in mESCs, namely, *RanBP17, XPO4, IPO7* and *Cse1L*. I also targeted *RanBP16 and KPNB1 (Importinβ1),* despite their moderate expressions in mESCs, because of *RanBP16* has high sequence identity with *RanBP17,* while *KPNB1* is widely known importer through its association with Importin-α. I speculated that LIF withdrawal after 48 h is necessary to enhance the effects of transfection on EB3 cells. Furthermore, I suggested that the 48 h window following the second transfection was critical, because it falls within the "temporal window" [26] where ES cells removed from pluripotency-promoting factors are still nonresponsive to differentiation-inducing agents. Therefore, at 48 h, we re-transfected the cells, changed the medium to LIF-withdrawn enriched DMEM, and maintained them for another 48 h before collection.

After 96 h, I observed that knockdown of *Cse1L* or *KPNB1* is lethal to mESCs (Fig. 4). This is not the case for the knockdown of the other target genes like *RanBP17, XPO4, IPO7* and *RanBP16* that resulted to viable cells after transfection. In addition, I observed signs of differentiation like spreading growth and appearance of flat polygonal cells in some colonies of mESCs transfected with Importin-β siRNA. However, majority of the colonies were morphologically similar to the control siRNA

treated mESCs. This result suggests that *Cse1L* and *KPNB1* are critically essential for mESC viability.

I successfully knocked down the select genes by using 2 different siRNAs in EB3 cells as analyzed by qPCR, with a non-targeting siRNA used as a control (Fig. 5A). I evaluated the effect of Importin-β knockdown on the ability of mESCs to maintain pluripotency by analyzing the changes in expression level of Nanog, Oct4 and Sox2; these transcriptional factors cooperatively maintain the regulatory network responsible for self-renewal and pluripotency in mESCs by coregulating large sets of genes and co-occupying many regulatory loci [30,31,32]. Interestingly, all siRNA treatments resulted in lower Nanog expression levels as compared to that in the control (Figs. 3B and 3E). Thomson *et al.* [28] demonstrated the necessity of Nanog downregulation for differentiation and lineage selection. Thus, my results indicated that knockdown of select Importin-β genes may predispose mESCs to cellular differentiation, suggesting that these genes play important roles in the maintenance of mESC pluripotency.

3.4. Effect of Importin-β knockdown on the expression of Oct4 and Sox2

Likewise, I observed Oct4 and Sox2 expression level changes in siRNA treated mESCs. As indicated in Figs. 3C-3E, an opposing expression pattern was observed for Oct4 and Sox2 from the different siRNA treatments. Knockdown of *RanBP17* and *XPO4* induced slightly higher Oct4 and lower Sox2 expression levels, while knockdown of *IPO7* resulted in lower Oct4 and higher Sox2 expression levels. Reduction of *RanBP16,* however, revealed comparable levels with the control for both genes. The observed variation in the Oct4/Sox2 expression levels precede cell fate selection as previously reported in mESCs [28].

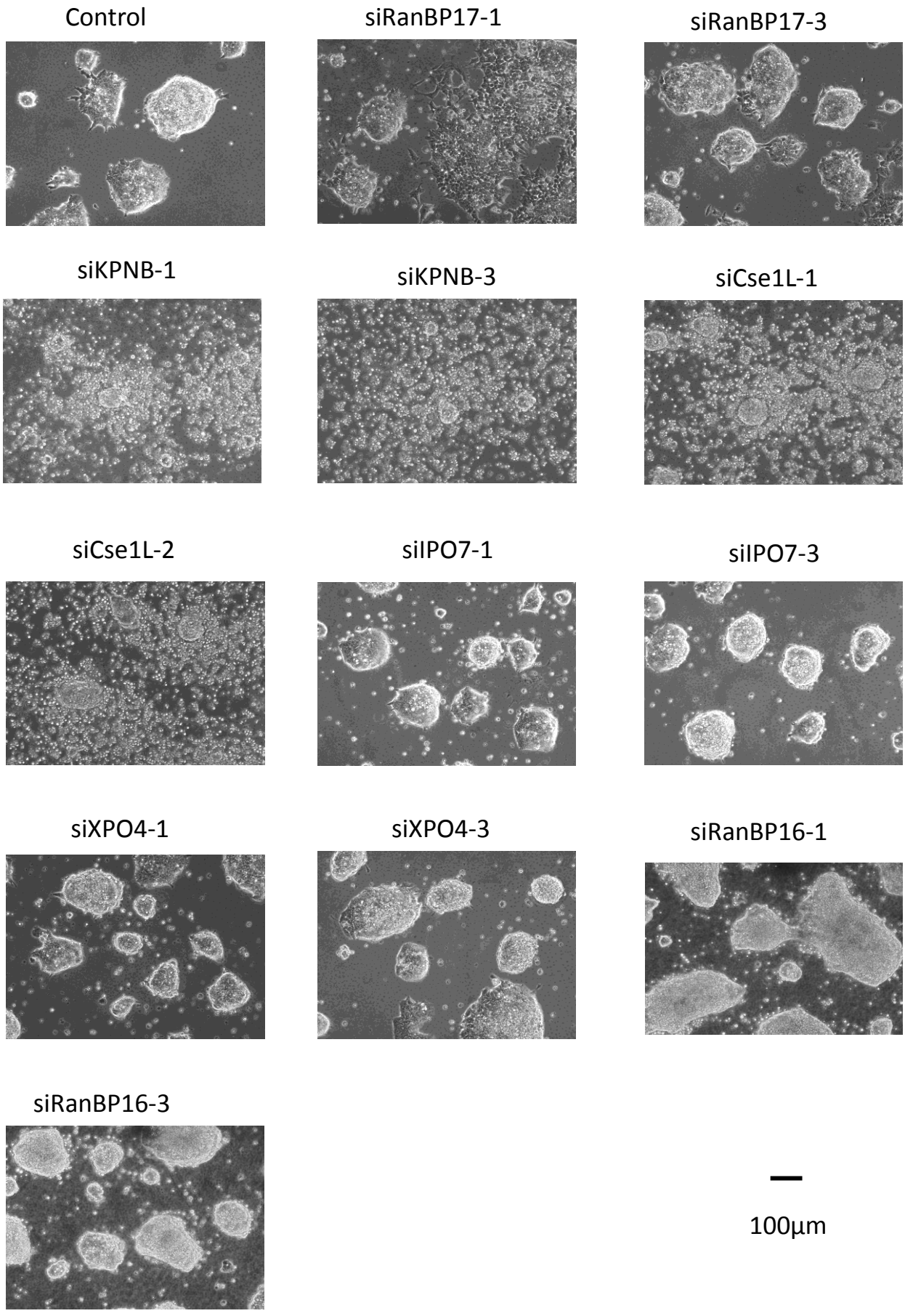


Figure 4. Phase contrast images of siRNA treated EB3 cells after 96 h incubation.

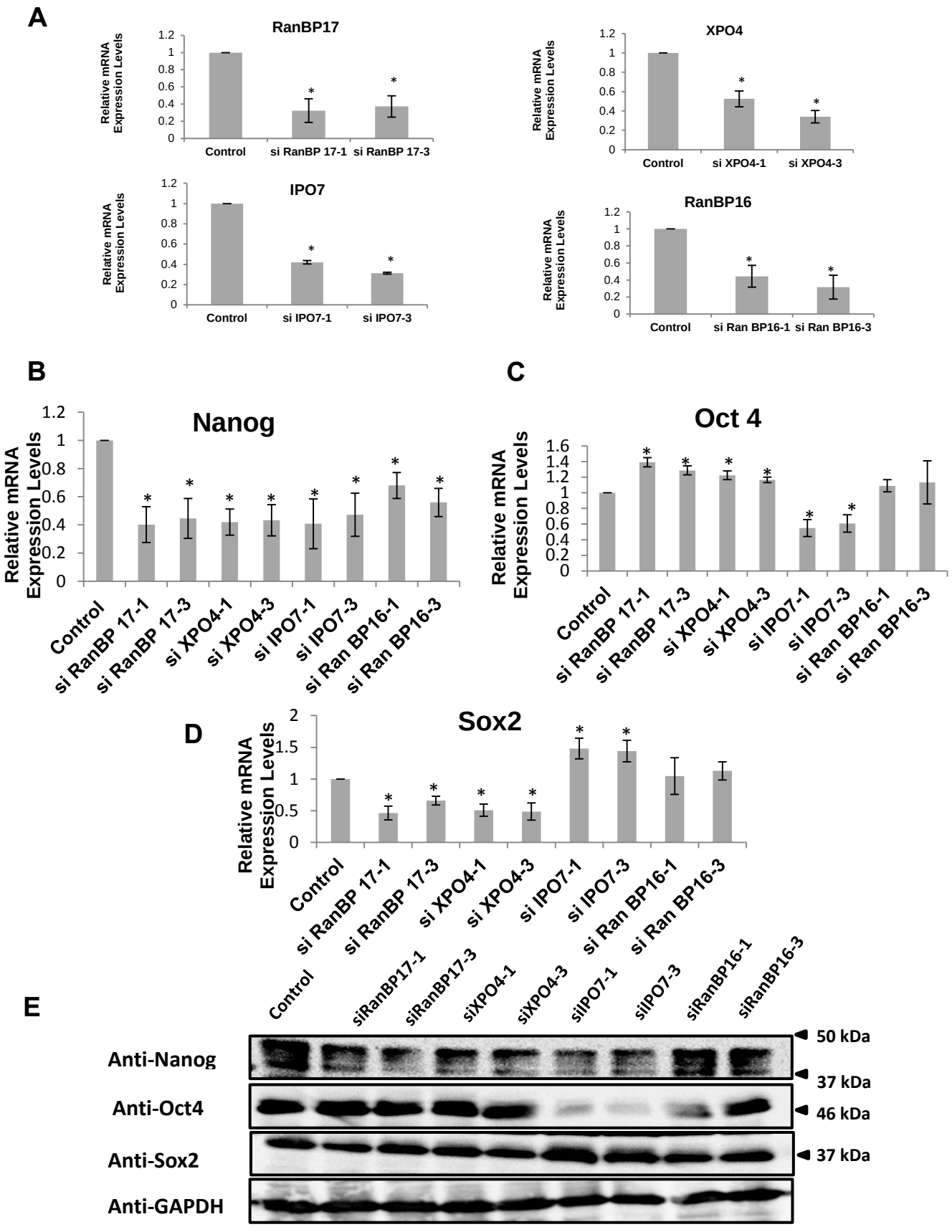


Figure 5. Effects of Importin-β knockdown on the expression of pluripotency markers. (A) Analysis of knockdown efficiency determined by qPCR analysis for *RanBP17, XPO4, IPO7,*

and *RanBP16* in siRNA-treated EB3 cells, using 2 variants of siRNA for each gene at 96 h incubation. All samples were normalized against GAPDH levels, and the expression level of each gene is presented as a fold change relative to the expression level of the same gene in non-targeting siRNA treated EB3 cells used as control. Significance was assessed and compared with the control using the unpaired Student's *t* test (*$p < 0.05$). Error bars represent SEM from 3 independent experiments. (B) qPCR analysis of Nanog in siRNA-treated EB3 cells incubated for 96 h in an enriched DMEM with LIF for the first 48 h and in LIF-withdrawn enriched DMEM for the next 48 h. All samples were normalized against GAPDH levels, and the expression of Nanog in siRNA-treated EB3 cells is presented as a fold change relative to its expression level in non-targeting siRNA treated EB3 cells used as control. Significance was assessed and compared with the control using unpaired Student's *t* test (*$p < 0.05$). Error bars represent SEM from 4 independent experiments. (C) qPCR analysis of Oct4 performed as in (B). (D) qPCR analysis of Sox2 performed as in (B). (E) Protein expression levels of Nanog, Oct4 and Sox2 in Importin-β siRNA-treated mESCs and in non-targeting siRNA-treated mESCs used as controls. Cell lysates (20 μg) were used for western blotting, and GAPDH was used as the loading control.

3.5. Effect of Importin-β knockdown on the expression of ME- or NE-specific markers

Considering the relationship between Importin-β expression levels and the changes in the expression levels of pluripotency markers (i.e. Nanog, Oct4 and Sox2), my results suggest that modulation of Importin-β expression may induce differentiation in mESCs. In order to see if the knockdown of Importin-βs could induce lineage specific differentiation, I further examined the activation and expression patterns of several differentiation markers including FGF5, Brachyury, FoxA2, Sox1 and Nestin. Except for FGF5, which is an early differentiation marker for primitive ectodermal differentiation, the other markers like Brachyury, FoxA2, Sox1 and Nestin are known to be germ layer specific, and their high expression levels in populations of differentiating cells indicate a definitive lineage fate. Brachyury and FoxA2 are main mesoendodermal (ME) regulators. Brachyury is specific for mesodermal

differentiation, whereas FoxA2 is a regulator of endodermal differentiation [28,29]. On the other hand, Sox1 and Nestin are readily detectable in early developing neuroectodermal (NE) cells [15,27,28]. However, my results showed either comparable or lower expression levels of FGF5, Brachyury, FoxA2, Sox1 and Nestin from the different Importin-β siRNA-treated cells in comparison with the control siRNA-treated cells (Figs. 6A-6E). Interestingly, the knockdown of *XPO4* or *RanBP16* in mESCs resulted in lower basal expression levels of Sox1compared to that in control siRNA treated cells, while reduction of *IPO7* resulted in reduced basal expression levels for both Sox1 and Nestin. Thus, knockdown of Importin-βs does not induce a lineage specific differentiation within a 48 h incubation period following LIF withdrawal. However, knockdown of Importin-βs within this “temporal window” predisposes the early differentiating mESCs toward a specific lineage.

3.6. ME-specific marker expression in CHIR99021-treated Importin-β knockdown cells

Next, I examined the effects of Importin-β knockdown on the cell differentiation process, which was promoted either by CHIR99021 or by RA treatment. As expected, I observed an upregulation of Brachyury in CHIR99021-treated cells, while the RA-treated cells showed very high expression levels of Sox1 and Nestin (Fig. 6B, 6D and 6E). In both treatments, relatively higher expression levels of FoxA2 were also recorded (Fig. 6C), which suggests that in either RA or CHIR99021 supplementation, there is a certain population of cells undergoing an endoderm differentiation, as described previously [29,33].

Next, I induced ME differentiation using CHIR99021 in siRNA-treated EB3 cells, and analyzed the resulting expressions of Brachyury and FoxA2. Knockdown of

RanBP16 resulted in a higher expression of Brachyury, which indicates the induction of mesodermal differentiation, while knockdown of other Importin-βs showed no obvious effects (Fig. 7A). High expression of FoxA2 was also observed from *RanBP16* knockdown cells (Fig. 7B), which further indicated enhanced endoderm differentiation. Comparatively similar to higher FoxA2 expression levels were also observed in *RanBP17, XPO4* and *IPO7* knockdown cells. Therefore, these results suggest that *RanBP16* hinders ME differentiation, while *XPO4* and *RanBP17* are associated with endodermal differentiation.

3.8. NE- and endoderm- specific marker expression in RA-treated Importin-β knockdown cells

I induced NE differentiation using RA in siRNA-treated EB3 cells. I also considered *IPO13* since it was highly expressed in NE cells (Fig. 3C). However, I failed to knockdown *IPO13* in EB3 cells using two variants of siIPO13 (nucleotide sequences are shown in Supplementary Table 1). The RA-treated cells with successful Importin-β knockdown were then analyzed for Sox1, Nestin and FoxA2 expression levels. Knockdown of *XPO4* or *IPO7* significantly decreased the expression levels of Sox1, while the rest of the siRNA treatments showed no obvious effects (Fig. 8A). Analogous with their Sox1 expressions, *XPO4 and IPO7* knockdown also resulted in very low expression levels of the NE marker Nestin (Fig. 8B). This indicates that reducing the levels of *XPO4 or IPO7* may inhibit NE differentiation in RA-treated mESCs.

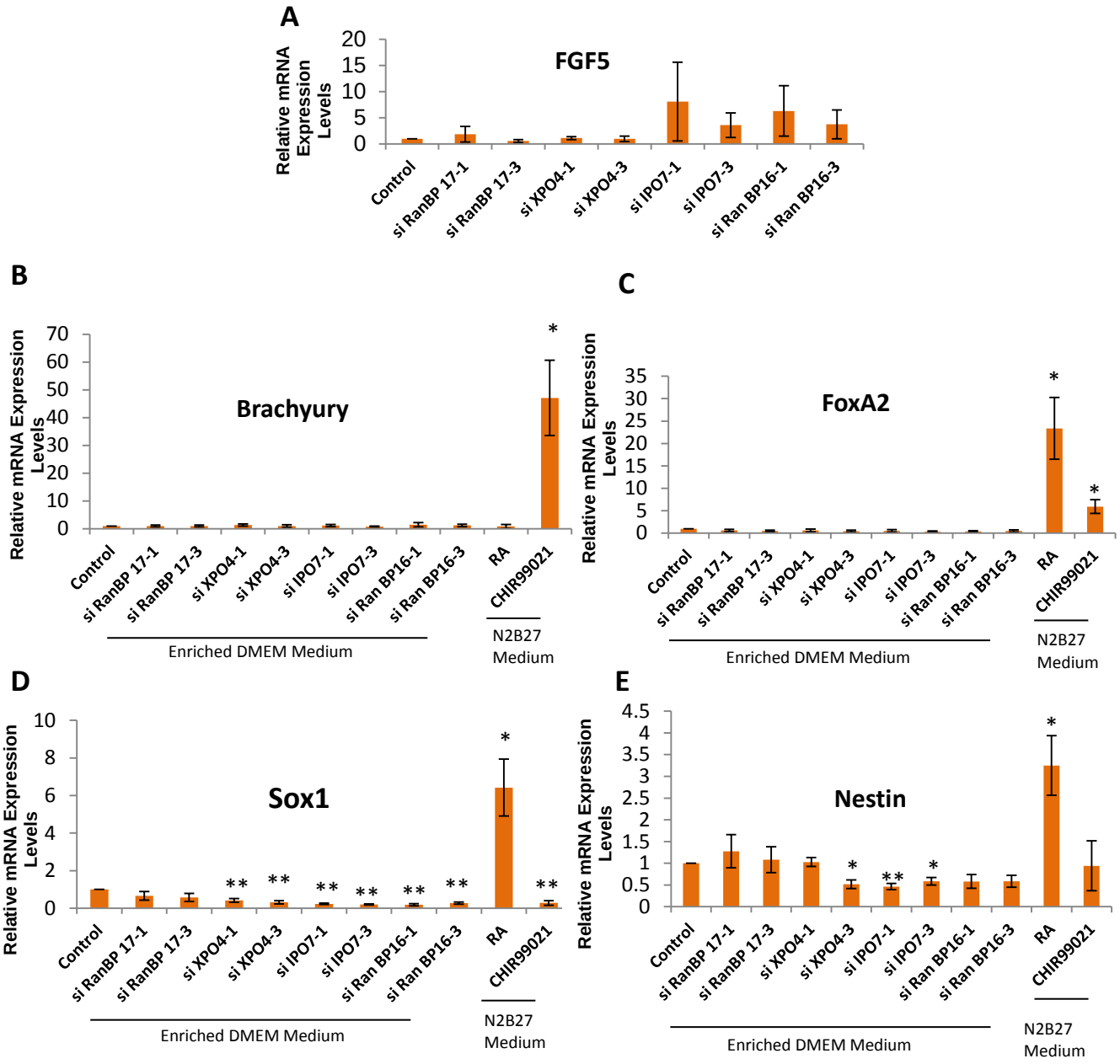


Figure 6. Effects of Importin-β knockdown on the expression of early differentiation markers and lineage-specific markers. (A) qPCR analysis of FGF5 in siRNA-treated EB3 cells incubated for 96 h in enriched DMEM with LIF for the first 48 h and in LIF-withdrawn enriched DMEM for the next 48 h. All samples were normalized against GAPDH levels, and the expression of FGF5 in siRNA-treated EB3 cells is shown as a fold change relative to its expression in non-targeting siRNA treated EB3 cells used as control. Significance was assessed and compared with the control using unpaired Student's *t* test (*$p < 0.05$; **$p < 0.01$). Error bars represent SEM from 4 independent experiments. (B) qPCR analysis of Brachyury performed as in (A) and from EB3 cells induced to differentiate using either CHIR99021 or RA maintained in N2B27 medium. (C) qPCR analysis of FoxA2 performed as in (B). (D) qPCR analysis of Sox1 performed as in (B). (D) qPCR analysis of Nestin performed as in (B).

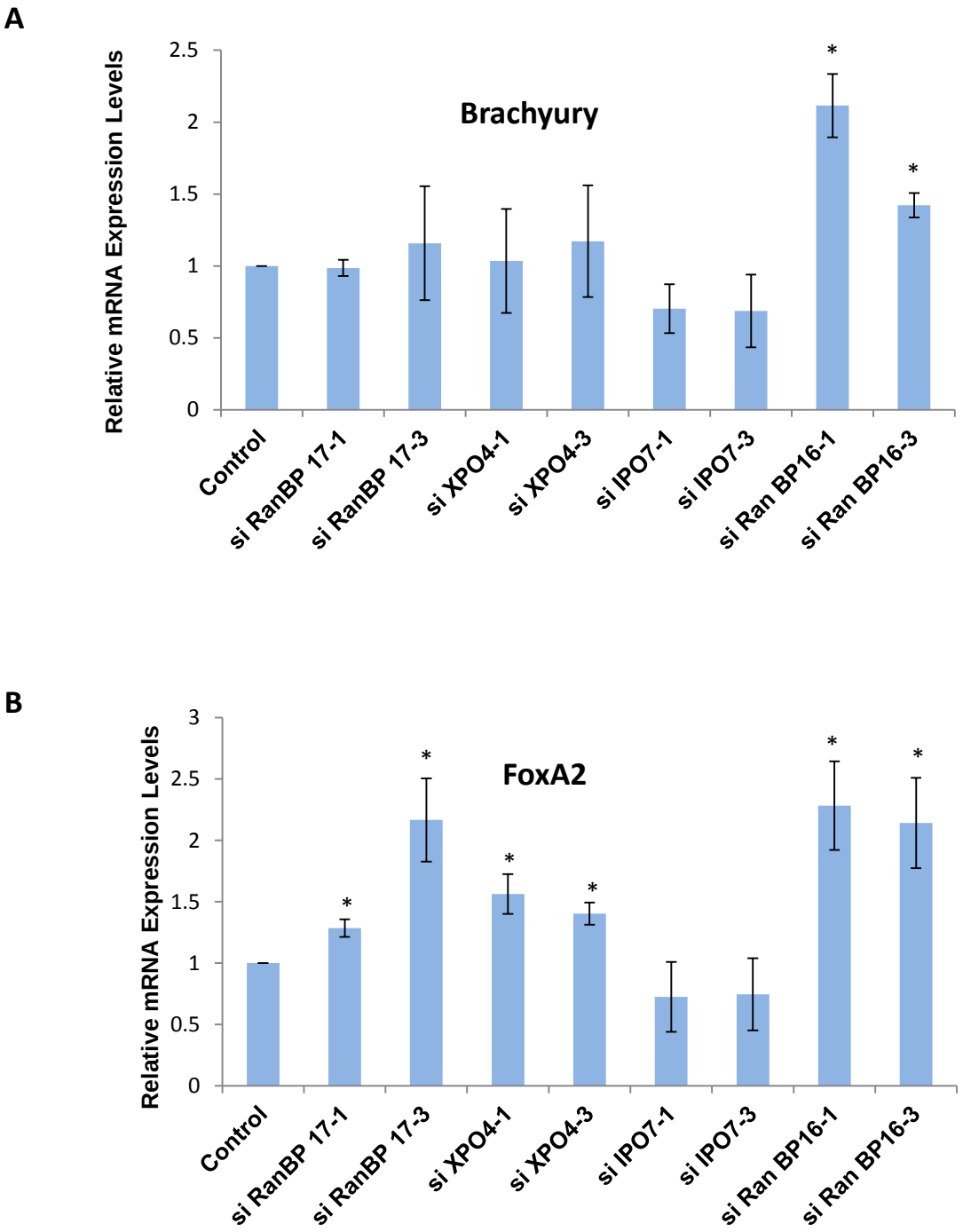


Figure 7. Expression of ME-specific markers from CHIR99021-treated Importin-β knockdown cells. (A) qPCR analysis of Brachyury in siRNA-treated EB3 cells incubated for 96 h in an enriched DMEM with LIF for the first 48 h and followed by CHIR99021-supplemented, LIF-withdrawn enriched DMEM for the next 48 h. All samples were normalized against GAPDH levels, and expression of Brachyury in siRNA-treated EB3 cells is shown as a fold change relative to its expression in non-targeting siRNA treated EB3 cells used as control. Significance was assessed and compared with the control using unpaired Student's *t* test (*$p < 0.05$; **$p < 0.01$). Error bars represent SEM from 4 independent experiments. (B) qPCR analysis of FoxA2 as performed in (A).

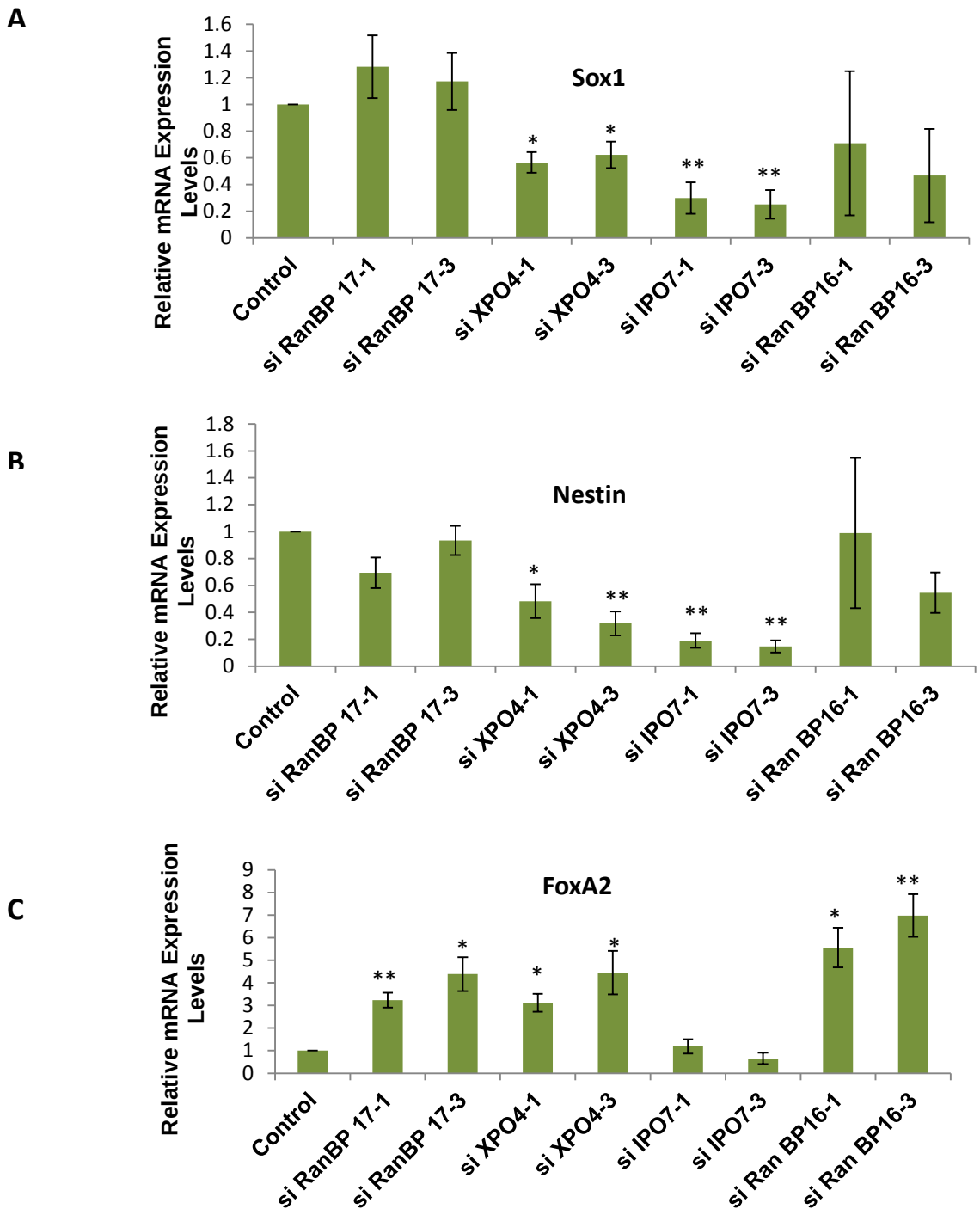
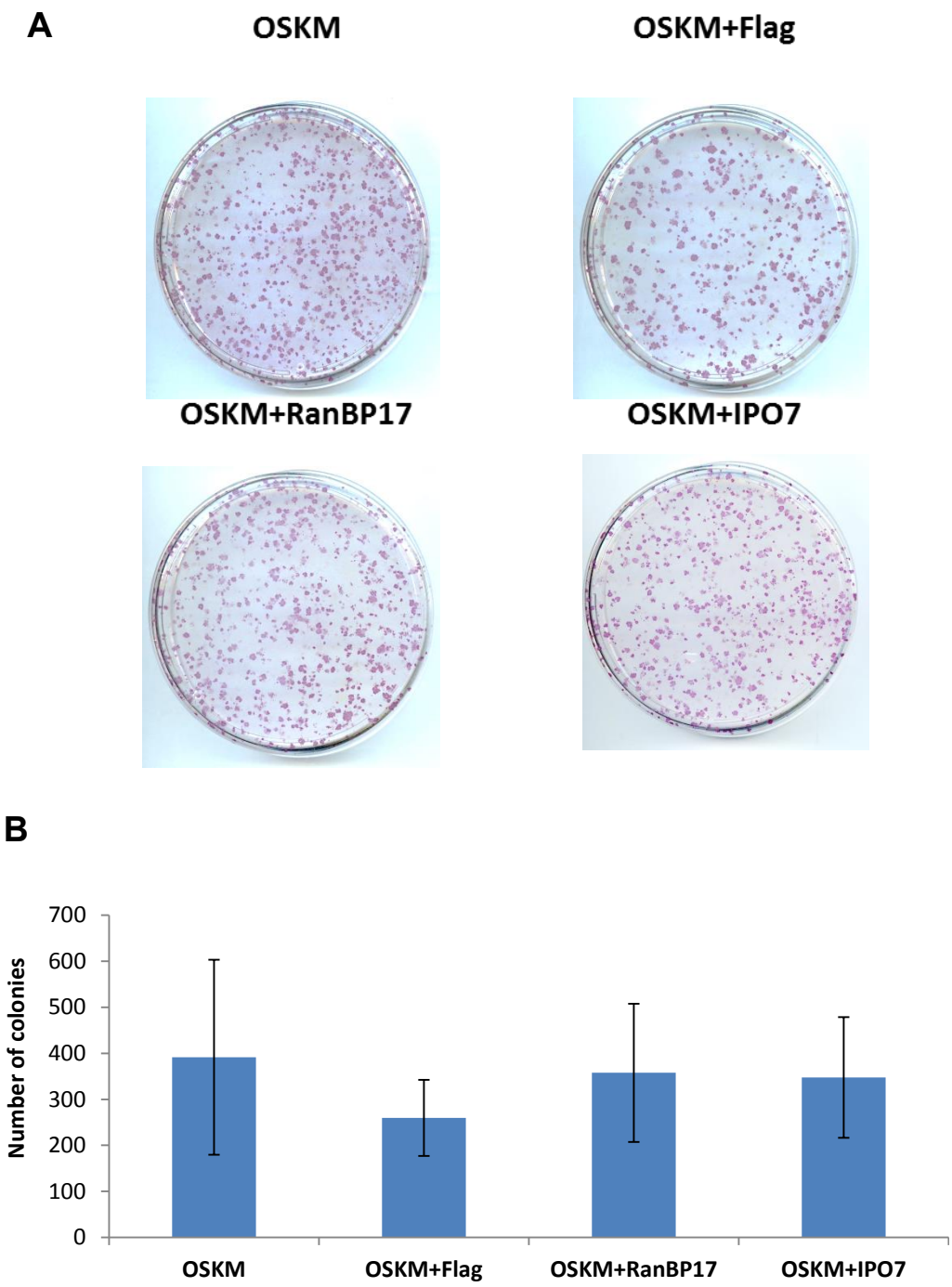


Figure 8. Expression of NE-and endoderm-specific markers from RA-treated Importin-β knockdown cells. (A) qPCR analysis of Sox1 in siRNA-treated EB3 cells incubated for 96 h in an enriched DMEM with LIF for the first 48 h, followed by retinoic acid (RA-) supplemented, LIF-withdrawn enriched DMEM for the next 48 h. All samples were normalized against GAPDH levels, and expression of Sox1 in siRNA-treated EB3 cells is shown as a fold change relative to its expression in non-targeting siRNA treated EB3 cells used as control. Significance was assessed and compared with the control using unpaired Student's *t* test (*$p < 0.05$; **$p < 0.01$). Error bars represent SEM from 4 independent experiments. (B) qPCR analysis of Nestin as performed in (A). (C) qPCR analysis of FoxA2 as performed in (A).

I also analyzed the FoxA2 expression level of RA treated Importin-β knockdown cells, since I observed a significant induction of FoxA2 expression in RA-treated EB3 cells grown in N2B27 medium (Fig. 6C). Interestingly, as seen in Fig. 8C, I noticed that the reduction in *RanBP16* caused a very high expression level of FoxA2, similar to what was observed in CHIR99021-treated cells (Fig. 7B). In addition, higher expression levels were also seen in *RanBP17* and *XPO4* knockdown cells. These findings further emphasized the involvement of *RanBP16, RanBP17* and *XPO4* in endodermal differentiation of mESCs.

3.9. Effect of Importin-β overexpression on cellular reprogramming

I further investigated the potential use of Importin-βs for cellular reprogramming. Specifically, I overexpressed *RanBP17* or *IPO7* in MEFs using respective pMXs retrovirus for *RanBP17* or *IPO7,* and simultaneously infected with a combination of pMXs retroviruses for mouse Oct4, Sox2, Klf4 and cMyc. I considered these two Importin-β genes since they were highly expressed in mESCs and I speculated that overexpressing either one of them may improve the efficiency of generating miPS cells from MEFs. As shown in Figures 9A and 9B, the overexpression of *RanBP17*or *IPO7* in reprogrammed MEFs showed lower colony counts after alkaline phosphatase staining. However, the reduction in the number of colonies seen in OSKM+RanBP17 or OSKM+IPO7 was not statistically significant from OSKM or OSKM+Flag (empty vector), which were used as controls. These results indicate that overexpression of *RanBP17*or *IPO7* in MEFs does not improve their reprogramming efficiency.



no significant difference was noted (*p value* > 0.05)

Figure 9. Reprogramming efficiency of MEFs 14 days post infection. (A) Representative images of plates after alkaline phosphatase staining. (B) Statistical analysis of miPS cell colony counts after alkaline phosphatase staining. Error bars represent SD from three independent experiments.

Collectively, my results demonstrate that Importin-β family members, such as *RanBP16, RanBP17, XPO4* and *IPO7,* are differentially involved in the lineage commitment of mESCs. However, some highly expressed Importin-βs in mESCs like *RanBP17* and *IPO7* have limited application in cellular reprogramming.

4. DISCUSSION

This is the first report on the mRNA expression patterns of Importin-β genes in mESCs and their differentiated germ layer cells (Figs. 2, 3C and 3D). Knockdown of highly expressed Importin-β genes in mESCs was performed to further understand their relationships to cell fate determination processes such as maintenance of pluripotency or lineage selection during differentiation.

4.1. Importin-β suppression promotes differentiation in mESCs

My data revealed a decrease in Nanog expression in Importin-β-knockdown cells (Fig. 5B). Nanog affects both pluripotency and differentiation propensity in mESCs [34, 35]. Nanog undergoes autorepressive regulation that is Oct4/Sox2-independent [36]. Moreover, Thomson *et al.* [28] suggested that Nanog downregulation could be an early and causal event for moving embryonic stem cells into the responsive state as an initial step towards differentiation. This suggests possible direct or indirect association of *RanBP17, XPO4, IPO7,* and *RanBP16* with the maintenance of pluripotency in mESCs and the suppression of at least one of these genes creates a condition that promotes differentiation. Protein-binding analysis of these Importin-β proteins will be required to further elucidate whether they associate with Nanog and other cargoes in mESCs, and how this is related to pluripotency.

4.2. Importin-β suppression in mESCs modulates a differential Oct4 and Sox2 level that leads to lineage-specific differentiation

My data also demonstrated variation in terms of Oct4 and Sox2 expression in response to Importin-β knockdown in mESCs (Fig. 5C-5E). Oct4 and Sox2 are transcription factors that, aside from their functions in the maintenance of pluripotency, have been reported to integrate external signals and control lineage selection. Specifically, Oct4 suppresses NE differentiation and promotes ME differentiation, while Sox2 hinders ME differentiation and promotes NE differentiation in mESCs and hESCs [28,37]. According to my results, *XPO4* knockdown caused a concomitant reduction in Sox2 expression. This observation is consistent with an earlier study that indicated that *XPO4* mediates Sox2 import [38]. The inability to import Sox2 after *XPO4* knockdown may in turn affect Sox2 expression, since Sox2 itself undergoes transcriptional autoregulation [39]. Consistent with this, a significant reduction in the expression of Importin-βs may change the nucleocytoplasmic traffic efficiency of Sox2 or other transcription factors involved in their transcriptional regulation. On the other hand, it has been shown that Importin-βs, aside from their transport roles, may interact directly with transcriptional factors and regulate their actions [24,40]. Depending on the Importin-β targeted, this would lead to high Oct4 and low Sox2 levels, or vice versa, thereby, predisposing mESCs to differentiate into ME or NE cells. However, I observed that the lineage-specific differentiation in knockdown cells is not yet readily detectable at this time. Nevertheless, following supplementation of inducing agents (i.e. CHIR99021 or RA) within the same “48 h window,” I was able to observe the effects of Importin-β knockdown on the lineage-specific differentiation of mESCs.

4.3. XPO4 and *RanBP17 suppression induce an endodermal differentiation in mESCs*

From this study, I found that reduction of *XPO4* and *RanBP17* in mESCs resulted in slightly higher Oct4 and lower Sox2 levels after 48 h incubation in a LIF withdrawn medium (Figs. 5C-5E), which is a condition favorable for ME differentiation. The specificity of *XPO4* knockdown cells to differentiate into endodermal cells was demonstrated by their selective up-regulation of FoxA2 compared with Brachyury, Sox1 and Nestin from the respective treatments (Figs. 7A-B and Figs. 8A-C). Similarly, a significant induction of FoxA2 expression was also observed in RA-treated *RanBP17* knockdown cells, however, only a comparable Brachyury level was observed following CHIR99021 treatment (Figs. 7A and 7B). These results indicated that reductions in both *XPO4* and *RanBP17* augment endodermal differentiation in mESCs.

4.4. RanBP16 suppression induces a ME differentiation in mESCs

Although, *RanBP16* knockdown cells showed no definite lineage specificity based on their Oct4 and Sox2 expression patterns (Figs. 5C-5E), the reduction of *RanBP16* in mESCs was shown to strongly promote ME differentiation as it led to very high expressions of Brachyury and FoxA2 following treatment with CHIR99021 and RA, respectively (Figs 7A-B and Fig. 8C). Collectively these findings suggest that *RanBP16* in mESCs may inhibit ME differentiation. Considering the different responses observed with *RanBP16* and *RanBP17* knockdown cells (Figs. 5C-5E, 7A-B and 8A-C), this study demonstrates the functional differences between these two homologous proteins.

4.5. XPO4 and IPO7 are essential for mESC differentiation into NE cells

As previously discussed, the knockdown of *XPO4* in mESCs concomitantly resulted in lower expressions of NE markers following RA treatment (Figs. 8A and 8B). Conversely, knockdown of *IPO7* resulted in lower Oct4 and higher Sox2 levels after a 48 h incubation period in a LIF withdrawn medium, which is a condition favorable for NE differentiation (Figs. 5C-5E). Surprisingly, *IPO7* reduction after RA treatment followed very low expressions of NE markers, Sox1 and Nestin (Figs 8A-C), suggesting that *XPO4* is important for the earlier stage of lineage commitment to NE, while *IPO7* is involved at the later stage of NE differentiation, although, the exact mechanism of their association in these cellular events is still unknown.

4.6. RanBP17 and IPO7 have limited potential in cellular reprogramming

As mentioned earlier, the overexpression of *RanBP17*or*IPO7* failed to improve the cellular reprogramming efficiency of MEFs to miPS cells (Figs. 9A-B). This is the case even though they were found to be highly expressed in mESCs and were also indicated to be associated in the maintenance of their pluripotency (Fig. 5B). This result suggests that highly expressed Importin-βs in mESCs may not be directly involved in the induction of cellular reprogramming of MEFs to miPS cells.

Taken together, my findings indicate that the expression patterns of Importin-β proteins in mESCs are distinct from their differentiated progenitor cells. The appropriate expression patterns of these proteins in mESCs are important in the maintenance of pluripotency and lineage choice during differentiation.

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Supplementary Table 1. Oligonucleotide sequences used in the study.

	Sequence	Purpose
GAPDH-F	AGGTCGGTGTGAACGGATTTG	Quantitative PCR
GAPDH-R	TGTAGACCATGTAGTTGAGGTCA	Quantitative PCR
XPO1-F	CCCTAATCAAGTGTGGGACAG	Quantitative PCR
XPO1-R	TGACTGTTTCAGGATCTTTCAGG	Quantitative PCR
XPO4-F	GGCAGCATCGAGTCACTG	Quantitative PCR
XPO4-R	CCCGAACATACTTTTGGAGGT	Quantitative PCR
XPO5-F	CGTCAGACATTTTGGCCTTC	Quantitative PCR
XPO5-R	CATGCTGTTCCATCGAAACTT	Quantitative PCR
XPO-t-F	CAGCAGCTCATCAGGGAGA	Quantitative PCR
XPO-t-R	GCTGCGGATTCTGCATCT	Quantitative PCR
KPNB1-F	AAATGGATTTGGCCATTGAG	Quantitative PCR
KPNB1-R	CGTAAAATTTGCTGGTGTGCT	Quantitative PCR
IPO4-F	CTGAAGGAAGACATGGAGGAGT	Quantitative PCR
IPO4-R	TCTGAAGCCACATCCACAAC	Quantitative PCR
IPO13-F	GGTGACTTCCGTCACACTCA	Quantitative PCR
IPO13-R	GCATAAATGAATCAACAATATCAGGA	Quantitative PCR
IPO5-F	GAGTGCATCAGCCTCATCG	Quantitative PCR
IPO5-R	GCATCACATCTGAAGCATCC	Quantitative PCR
IPO7-F	TCAGAACAACTGGATTTACCTGTG	Quantitative PCR
IPO7-R	CGATCAGGCCAATATTGTGTTA	Quantitative PCR
IPO8-F	TTCACAATGTGCAGGAAGGT	Quantitative PCR
IPO8-R	AGCATAGCACTCGGCATCTT	Quantitative PCR
IPO11-F	CTCAGCTTTGGCTTTGCTTT	Quantitative PCR
IPO11-R	TCCACAAAATTTATCTTGGATAACAC	Quantitative PCR
RanBP6-F	TTTGTCACCTTCCCCCTATTT	Quantitative PCR
RanBP6-R	CATCTTGGCACAAAATATACCG	Quantitative PCR
RanBP16-F	TCCTCGAGAGAGGAAGTTCG	Quantitative PCR
RanBP16-R	GCTTGGTAAGGCATGTAGCTG	Quantitative PCR
RanBP17-F	GGGACTCACCACACTTGACA	Quantitative PCR
RanBP17-R	GACCATGTAGTCTAAACTTGTACAGCA	Quantitative PCR
Cse1L-F	AACAAACCTTTTCAAAGCTCTCAC	Quantitative PCR
Cse1L-R	TCTTGCAGGAGGGAGAAACT	Quantitative PCR
TRN1-F	GCTTGAAGTCCGGATGGAT	Quantitative PCR
TRN1-R	GGGTTCTCTGCAGCATGTACT	Quantitative PCR
TRN2-F	CAAGGAAGTCCTGGCCTCT	Quantitative PCR
TRN2-R	TTCATCCCATTCACGAGGA	Quantitative PCR
TRN3-F	CTGTCGGCAGGGGTTATTAG	Quantitative PCR
TRN3-R	AGGAGCTGAAAGGTGGGAAT	Quantitative PCR
RanBP9-F	AGAAGCAGAGCAGACAAAAGC	Quantitative PCR

	Sequence	Purpose
RanBP9-R	GCCTAGAGCAAGCATGCAG	Quantitative PCR
XPO6-F	AAGGAAGCGGGAGATAGAGG	Quantitative PCR
XPO6-R	AAGCAGAATCTCCAAGCTCCTA	Quantitative PCR
RanBP10-F	ACAGCAGTACCAGTAACCAGGAG	Quantitative PCR
RanBP10-R	CCAGCACACCATTGGGATA	Quantitative PCR
endo-Oct4-F	TAGGTGAGCCGTCTTTCCAC	Quantitative PCR
endo-Oct4-R	GCTTAGCCAGGTTCGAGGAT	Quantitative PCR
endo-Sox2-F	AGGGCTGGGAGAAAGAAGAG	Quantitative PCR
endo-Sox2-R	CCGCGATTGTTGTGATTAGT	Quantitative PCR
endo-nanog-F	TTGCTTACAAGGGTCTGCTACT	Quantitative PCR
endo-nanog-R	ACTGGTAGAAGAATCAGGGCT	Quantitative PCR
endo-FGF5-F	AAAACCTGGTGCACCCTAGA	Quantitative PCR
endo-FGF5-R	CATCACATTCCCGAATTAAGC	Quantitative PCR
Brachyury-F	CAGCCCACCTACTGGCTCTA	Quantitative PCR
Brachyury-R	GAGCCTGGGGTGATGGTA	Quantitative PCR
FoxA2-F	GAGCAGCAACATCACCACAG	Quantitative PCR
FoxA2-R	CGTAGGCCTTGAGGTCCAT	Quantitative PCR
Sox1-F	GAGTGGAAGGTCATGTCCGAGG	Quantitative PCR
Sox1-R	CCTTCTTGAGCAGCGTCTTGGT	Quantitative PCR
Nestin-F	TCCCTTAGTCTGGAAGTGGCTA	Quantitative PCR
Nestin-R	GGTGTCTGCAAGCGAGAGTT	Quantitative PCR
Brachyury-F	AAGGAACCACCGGTCATC	RT-PCR
Brachyury-R	GTGTGCGTCAGTGGTGTGTAATG	RT-PCR
Sox1-F	CAAGATGCACAACTCGGAGA	RT-PCR
Sox1-R	GTCCTTCTTGAGCAGCGTCT	RT-PCR
Actin-F	TTCCTTCTTGGGTATGGAAT	RT-PCR
Actin-R	GAGCAATGATCTTGATCTTC	RT-PCR
siRanBP17-1	GAAACUACAUCCUGAAUUA	siRNA
siRanBP17-3	CGAGAAGUAUUUCAGUGAA	siRNA
siXPO4-1	UGACAAGCAUUUCCAUAAA	siRNA
siXPO4-3	AGACUUACCUCCUGGUAGA	siRNA
siIPO7-1	UGAUGCCUCUUCUACAUAA	siRNA
siIPO7-3	AUAGGGAUGUACCUAAUGA	siRNA
siRanBP16-1	GCAAGAUGAUAACAAUGUA	siRNA
siRanBP16-3	CCAGCAAGAUGAUAACAAU	siRNA
siCse1L-1	UCACAUACUUCCUGAUUUA	siRNA
siCse1L-2	GCAAUAUGCUUGUCUAUAA	siRNA
siKPNB-1	CCAGCAAAUUUUACGCCAA	siRNA
siKPNB-3	GGAGGAGCCUAGUAACAAU	siRNA

	Sequence	Purpose
siIPO13-1	UCAUCCUGAUAUUGUUGAU	siRNA
siIPO13-3	GCGACUGGAUGUCAAAGCU	siRNA
IPO7F (BamHI)	CATGGGATCCACCATGGACCCCAACACCATCAT	Cellular reprogramming
IPO7R (SalI)	GATCGTCGACTCAATTCATCCCCGGTGCTG	Cellular reprogramming
RanBP17F (BamHI)	CATGGGATCCACCATGGCGCTGCACTTTCAGAG	Cellular reprogramming
RanBP17R (SalI)	GATCGTCGACTCAGCTCATCATGTCCAGGCT	Cellular reprogramming

ACCOMPLISHMENTS

Presentation

1. **Sangel Percival**, Oka Masahiro and Yoneda Yoshihiro. Comparative Expression/Functional Analysis of Karyopherin-βs in Mouse Embryonic Stem Cells and Differentiated Cells.

   2013 International Conference on Life Science and Biological Engineering (Tokyo, Japan), March 15-17, 2013
2. Oka Masahiro, **Sangel Percival**, Kimura Hiroshi and Yoneda Yoshihiro. Functional Analysis of Nup98-Hox Fusion.

   Annual Meeting of Molecular Biology Society of Japan, December 2013