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Effect of Dual-Specificity Protein Phosphatase 5 on Pluripotency Maintenance and Differentiation of Mouse Embryonic Stem Cells

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ABSTRACT

The MAPK/Erk signaling pathway is considered as a key regulator of the pluripotency and differentiation of embryonic stem (ES) cells, while dual-specificity protein phosphatases (DUSPs) are negative regulators of MAPK. Although DUSPs are potential embryogenesis regulators, their functions in the regulation of ES cell differentiation have not been demonstrated. The present study revealed that Dusp5 was expressed in mouse ES (mES) cells and that its expression was correlated with the undifferentiated state of these cells. Exogenous Dusp5 expression enhanced mES cell clonogenicity and suppressed mES cell differentiation by maintaining *Nanog* expression via the inhibition of the Erk pathway. Following *Dusp5* knockdown, *Nanog* and *Oct4* expression was significantly attenuated and the Erk signaling pathway was activated. Additionally, EBs derived from *Dusp5* knockdown mES cells (KDEBs) exhibited a weak adherence capability, very little outgrowth, and a reduction in the number of epithelial-like cells. The expression of *Gata6* (an endodermal marker) and *Flk1* and *Twist1* (mesodermal markers) was inhibited in KDEBs, which indicated that Dusp5 influenced the differentiation of these germ layers during EB development. Collectively, this study suggested that Dusp5 plays an important role in the maintenance of pluripotency in mES cells, and that Dusp5 may be required for EB development. J. Cell. Biochem. 112: 3185–3193, 2011.

KEY WORDS: EMBRYONIC STEM CELLS; DUSP5; PLURIPOTENCY; DIFFERENTIATION

Pluripotent mouse embryonic stem (mES) cells, maintained artificially on feeder cells, such as mouse embryonic fibroblasts (MEFs), with fetal calf serum, were first described in 1981 [Evans and Kaufman, 1981; Martin, 1981]. ES cells are defined by their capacity for unlimited symmetrical self-renewal and for their ability to differentiate into all derivatives of the three primary germ layers: Ectoderm, endoderm, and mesoderm. In recent decades, various cytokines, transcription factors, and signaling networks have been suggested to play crucial roles in the maintenance of ES cell pluripotency [Marshak et al., 2001].

Recently, the mitogen-activated protein kinase (MAPK) signaling pathway has received great attention in the field of pluripotency research. Although the activation of MAPK/Erk signaling is not essential for ES cell self-renewal, the suppression of Erk signaling can prevent ES cells from differentiating. Burdon et al. [1999] were the first to demonstrate that the inhibition of ERK activation enhanced ES cell self-renewal. Over the next decade, many reports showed that the inhibition of Erk signaling allowed ES cells to retain their ability to self-renew, which facilitated the establishment of ES cell lines [Buehr and Smith, 2003; James et al., 2003; Feng, 2007; Kunath et al., 2007; Nichols et al., 2009]. Ying et al. [2008] described a new cell culture medium capable of establishing and maintaining ES cell lines more efficiently than other traditional media types. This "3i" medium is defined by the presence of three inhibitors including the fibroblast growth factor receptor (FGFR) inhibitor, PD184352, the Erk cascade inhibitor, SU5402, and the glycogen synthase kinase-3 (GSK3) inhibitor, CHIR99021. Taken together, these findings suggest that Erk signaling plays a dominant role in embryogenesis (for reviews see Binétruy et al., 2007; Wray et al., 2010).

Abbreviations: EB, embryoid body; ES, embryonic stem cell; KDEB, EB derived from *Dusp5* knockdown mES D3 cells; OED5, mES D3 cells over-expressing the Dusp5 protein; OEE, mES D3 cells over-expressing the EGFP protein; Scr, scramble shRNA-treated mES D3 cells; ScrEB, EB derived from Scr cells; SHD5-pool, *Dusp5* knockdown mES D3 cells; WT, wild type mES D3 cells; WTEB, EB derived from WT cells.

Additional supporting information may be found in the online version of this article.

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Dual-specificity protein phosphatase 5 (Dusp5, also known as HVH3 and B23) belongs to the DUSP family (also widely known as MAPK phosphatases or MKPs), which dephosphorylate both the phosphothreonine and phosphotyrosine residues on activated MAPKs. This phosphatase is induced rapidly by both heat shock and serum [Ishibashi et al., 1994; Kwak and Dixon, 1995], and is degraded quickly in mammalian somatic cells [Kucharska et al., 2009]. Unlike Dusp1 (MKP1), Dusp4 (MKP2), and Dusp10 (MKP10) [Liu et al., 2007], Dusp5 inactivates Erk2 but not Jun N-terminal protein kinase or p38 MAP kinase. Dusp5 is also involved in the nuclear translocation of Erk2 in vivo [Mandl et al., 2005], serving as a negative feedback mechanism for the control of Erk signaling.

In *Xenopus* early development, *Dusp5* mRNA levels increase between blastula stages 8.5–9, and together with Dusp1, it is a negative regulator of fibroblast growth factor (FGF) signaling [Branney et al., 2009]. In the zebrafish embryo, Dusp5 is expressed in angioblasts and is essential for vascular development in vivo, while the loss of Dusp5 function causes the apoptosis of endothelial cells in vitro [Pramanik et al., 2009]. To date, there have been few studies investigating the function of Dusp5 in mammalian embryonic development, and the mechanism by which DUSP proteins regulate embryogenesis via the MAPK/Erk pathway is still unclear.

In this study, we have analyzed the functions of Dusp5 in mES cells. Our results show that Dusp5 expression correlates with the undifferentiated state of mES cells, and decreases dramatically during embryoid body (EB) formation. Mouse ES cells over-expressing Dusp5 possessed enhanced self-renewal ability, while *Dusp5* knockdown resulted in the reduced expression of *Nanog* and *Oct4*, though it did not influence mES cell proliferation significantly. EBs derived from *Dusp5* knockdown cells (KDEBs) showed some unusual characteristics, such as poor adherence, little outgrowth and fewer epithelial-like cells. In addition, the gene expression levels of *Gata6* (an endoderm marker) and *Flk1* and *Twist1* (mesodermal markers) were attenuated and delayed during EB development following *Dusp5* knockdown. Our work suggests that Dusp5 is a potential candidate for an anti-differentiation regulator of mES cells, and also that it is involved in the regulation of EB development, probably via the Erk signaling pathway.

MATERIALS AND METHODS

CELL CULTURE

The mES D3 and HEK293T cell lines were purchased from ATCC (Manassas, VA). The D3 cells were cultured in ES cell medium (DMEM supplemented with 15% fetal bovine serum, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 1× NEAA, and 10 ng/ml LIF) on feeder cells as described previously [Gu et al., 2010], or were grown on gelatin-coated tissue culture plates without feeder cells. The HEK293T cells were cultured in 293 cell medium (DMEM supplemented with 10% fetal bovine serum).

PLASMID CONSTRUCTION

The shRNA plasmids modified with enhanced green fluorescent protein (EGFP), were constructed from pLKO.1-TRC [Moffat et al., 2006] as described previously [Gu et al., 2010].

The plasmids, pCS and PUW-OS were purchased from Addgene (Cambridge, MA). The human ubiquitin C (hUbc) promoter was amplified by PCR from the plasmid PUW-OS, and then inserted into the *Bam*HI–*Eco*RI restriction sites of the plasmid, pCS, to form pCS-UBC. *Dusp5* open reading frame (ORF) cDNA was cloned by PCR from mES D3 cDNA, and then inserted into the *Xho*I–*Kpn*I restriction sites of the plasmid, pCS-UBC, to form pCS-UBC-Dusp5. The plasmid, pCS-UBC-EGFP, was constructed using the same strategy and was used as a control. The PCR primers carrying the restriction sites mentioned above are listed in Table I.

TRANSIENT TRANSFECTION, LENTIVIRAL PRODUCTION, AND INFECTION

Cells were transfected using polyethylenimine (PEI), linear, MW 25,000 (Polysciences, Inc., Oakville, ON, Canada). Briefly, cells were cultured overnight until 50–80% confluent on the day of the experiment. Cells were transfected using a 12:1 ratio (μg) of PEI to

TABLE I. Oligonucleotide Primers Used in This Study

Gene	Primer	Sequence (5′–3′)[a]	Purpose
EGFP	LEGFPF	CGGGATTCTCTTTCCTGCGTTATCCCCT	Amplifying CMV-EGFP
	LEGFPR	GGGGTACCTTACTTGTACAGCTCGTCCATG	Amplifying CMV-EGFP or EGFP
	EGFPF3	CCGCTCGAGATGGTGAGCAAGGGCGAG	Amplifying EGFP
hUBC	hUbcF	CGGGATCCCCTAACCCGTGTCGGCTC	Amplifying hUbc promoter
	hUbcR	GCTCCGTCTCCATCATGTTAT	Amplifying hUbc promoter
Gapdh	mGAPDHF	CATGGAGAAGGCCGGGG	RT-PCR analysis
	mGAPDHR	CTGCACCACCAACTGCTT	RT-PCR analysis
Dusp5	DUSP5F4	CCGCTCGAGCTATGAAGGTCACGTCGCTCGACG	Amplifying Dusp5
	DUSP5R4	GGGGTACCTCAGCAGGATGTGGCTGTGGC	Amplifying Dusp5
	mDUSP5F5	CCACTTTCAAGAAGCAATAGA	Amplifying Exogenous Dusp5
	PCSR	CTGCCTTGTAAGTCATTGGT	Amplifying Exogenous Dusp5
	DUSP5qF	TGCACCACCCACCTACACTA	qRT-PCR analysis
	DUSP5qR	ATGTCAGCAGTGTGGCTGTC	qRT-PCR analysis
Nanog	NanogqF	TTGCTTACAAGGGTCTGCTACT	qRT-PCR analysis
	NanogqR	ACTGGTAGAAGAATCAGGGCT	qRT-PCR analysis
β-actin	β-actinqF	AGTGTGACGTTGACATCCGTA	qRT-PCR analysis
	β-actinqR	GCCAGAGCAGTAATCTCCTTCT	qRT-PCR analysis
Flk1	Flk1qF	TTTGGCAAATACAACCCTTCAGA	qRT-PCR analysis
	Flk1qR	GCAGAAGATACTGTCACCACC	qRT-PCR analysis
Gata6	Gata6qF	TTGCTCCGGTAACAGCAGTG	qRT-PCR analysis
	Gata6qR	GTGGTCGCTTGTGTAGAAGGA	qRT-PCR analysis

[a]Restriction endonuclease sites used in cloning are underlined.

DNA. Plasmids were incubated with diluted PEI in Opti-MEM for 30 min at room temperature, followed by transfection of cells.

Virus production and infection were performed as described previously [Gu et al., 2010].

CLONOGENIC ASSAY

Mouse ES cells were seeded in 24-well cell culture plates on feeder cells at a density of 500 cells/well and then allowed to grow for 4 days. Alkaline phosphatase (ALP) staining was performed with an ALP assay kit (Sigma). Macroscopic pictures were taken using a digital camera (Panasonic DMC-ZS1) and the number of ALP positive clones was counted under a phase contrast microscope. The results were expressed as statistical averages of three independent wells.

COLONY DIFFERENTIATION ASSAY

Mouse ES cells were cultured initially on gelatinized tissue culture plates (300–500 cells/ml) in ES cell medium containing LIF. After 4 days, the culture medium was changed with LIF-free medium for an additional 2 days and colonies were stained using an ALP assay kit and photographed. Colonies showed flattened, fibroblast-like morphology, and with low ALP staining were counted as clearly differentiated colonies.

EMBRYOID BODY (EB) DIFFERENTIATION

The hanging drop method was used for EB formation as described previously [Höpfl et al., 2004] with slight modifications. The cell density was adjusted to 50,000 cells/ml in differentiation medium (DMEM supplemented with 15% fetal bovine serum, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine and 1× NEAA), and then hanging drops (20 μl/droplet) were placed on the lid of a Petri dish filled with phosphate-buffered saline (PBS), and the cells were cultivated for 2 days. After a further 2-days cultivation in a bacterial dish, EBs were plated onto gelatin-coated tissue culture plates for morphological analysis and RNA sample collection.

For spontaneous cardiomyocyte differentiation, EBs were formed by the hanging drop method. After being cultured in suspension for a further 3 days, these 5-day-old EBs were plated on to gelatin-coated tissue culture plates. The generation of contracting cells present in the EB-derived populations was detected under a phase contrast microscope.

MONOLAYER DIFFERENTIATION

Monolayer differentiation was performed as described previously [Gu et al., 2010] with slight modifications. The cells were seeded at a density of 5,000–7,000 cells/cm^2 on gelatin-coated tissue culture plates in differentiation medium. RNA samples were collected on different days.

QUANTITATIVE RT-PCR (QRT-PCR)

RNA was prepared and first strand cDNA was synthesized as described previously [Gu et al., 2010]. For qRT-PCR, the analysis of mRNA levels was performed with SYBR Green Reagents (TOYOBO) using an iQ5 Multicolor Real-time PCR Detection System, and all mRNA levels were normalized to β-actin. Intergroup differences were assessed by the Student's *t*-test using the StatView 5.0 program (SAS Institute Inc., Cary, NC), $^{*}P < 0.05$, $^{**}P < 0.01$. The column charts were drawn using Origin 8.0 software (OriginLab, MA). The PCR primers using in this study are listed in Table I.

WESTERN BLOT

Western Blot analysis was performed as described previously [Tan et al., 2011]. Antibodies against Erk-1/2, phosphorylated Erk-1/2 (Thr202 and Thr204) (Cell Signaling), Dusp5 (Santa Cruz, CA), β-actin (Santa Cruz), and Gapdh (Santa Cruz) were used in this study.

RESULTS

DUSP5 TRANSCRIPTION PROFILES IN mES D3 CELLS AND IN EB DEVELOPMENT

To determine the relationship between Dusp5 and pluripotency, undifferentiated mES D3 cells and EBs, at different stages of development, were harvested and the *Dusp5* transcript characteristics were analyzed during ES cell differentiation. Transcript levels were measured by qRT-PCR analysis, and the results showed that *Dusp5* was expressed in undifferentiated mES cells (Fig. 1A). During EB formation and development *Dusp5* expression was reduced fourfold initially, then gradually increased to half the level seen in undifferentiated mES cells in 6-day EBs, and continued to increase in 12-day EBs. A similar result was observed during monolayer differentiation (Fig. 1B). These findings suggested that Dusp5 is a pluripotency-associated protein, which may also be required for EB development.

EXOGENOUS DUSP5 MAINTAINS PLURIPOTENCY OF mES D3 CELLS

Mouse ES D3 cells over-expressing the Dusp5 protein (OED5 cells), and mES D3 cells over-expressing the EGFP (OEE cells) were obtained (SI Text).

To examine the function of exogenous Dusp5, a clonogenic assay was performed. Figure 2A and B reveal that cells over-expressing Dusp5 showed increased mES cell clonogenicity. Next, a colony differentiation assay was used to determine the differentiation ability of the mES cells. Interestingly, a considerable number of OED5 cell colonies remained morphologically undifferentiated and retained a densely packed shape with deeper ALP staining, whereas all of the OEE and wild type (WT) colonies exhibited the flattened colony morphology normally associated with differentiation (Fig. 2C). The percentage of clearly differentiated colonies on the plates was calculated, and the results showed that only 12.3% of the OED5 cell colonies had differentiated compared to 30.5% of OEE and 32.6% of WT colonies (Fig. 2D), indicating a significantly lower rate of differentiation for OED5 cells. Similar results were observed in three independent experiments.

To characterize further the role played by exogenous Dusp5 in the promotion of pluripotency, qRT-PCR was used to analyze the expression levels of the ES cell markers, *Nanog* and *Oct4*. When cultured in conditions that maintained the cells in the undifferentiated state, no detectable difference was found between the three cell lines; however, OED5 cells exhibited a significant increase in *Nanog* expression when LIF was reduced to 5 ng/ml from 10 ng/ml. Likewise, the expression of *Oct4* showed an upward trend (Fig. 3A).

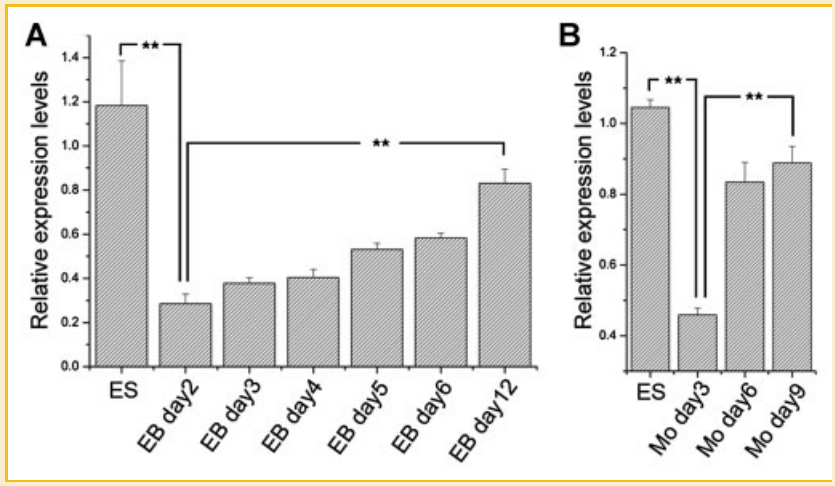

Fig. 1. Transcription characteristics of *Dusp5* during mES cell differentiation. A: *Dusp5* transcription levels were analyzed by qRT-PCR (n = 4) in undifferentiated mES cells and embryoid bodies (EB). B: *Dusp5* transcription levels were analyzed by qRT-PCR (n = 4) in undifferentiated mES cells and monolayer differentiated mES cells (Mo). The gene expression was normalized against a β-actin control. Error bar represents the mean values ± S.D. from four independent experiments. $^{**}P < 0.01$.

The phosphorylation of Erk in these cells was tested by Western blot analysis with antibodies against phosphorylated and unphosphorylated Erk. OED5 cells showed weaker immunoblot signals for phosphorylated Erk1 and Erk2 compared to OEE cells and WT cells (Fig. 3B). These results demonstrated that exogenous Dusp5 maintained *Nanog* expression via the negative regulation of Erk activity in mES cells.

DUSP5 KNOCKDOWN RESULTS IN REDUCTION OF NANOG AND Oct4 TRANSCRIPTION, BUT DOES NOT AFFECT THE PROLIFERATION OF mES D3 CELLS

Mouse ES D3 cells, subjected to shRNA targeting of *Dusp5* mRNA (SHD5-pool cells), and mES D3 cells treated with scramble shRNA (Scr cells) were obtained (SI Text). Two isolated clones were obtained from *Dusp5* knockdown cells (SHD5-e5, SHD5-g2).

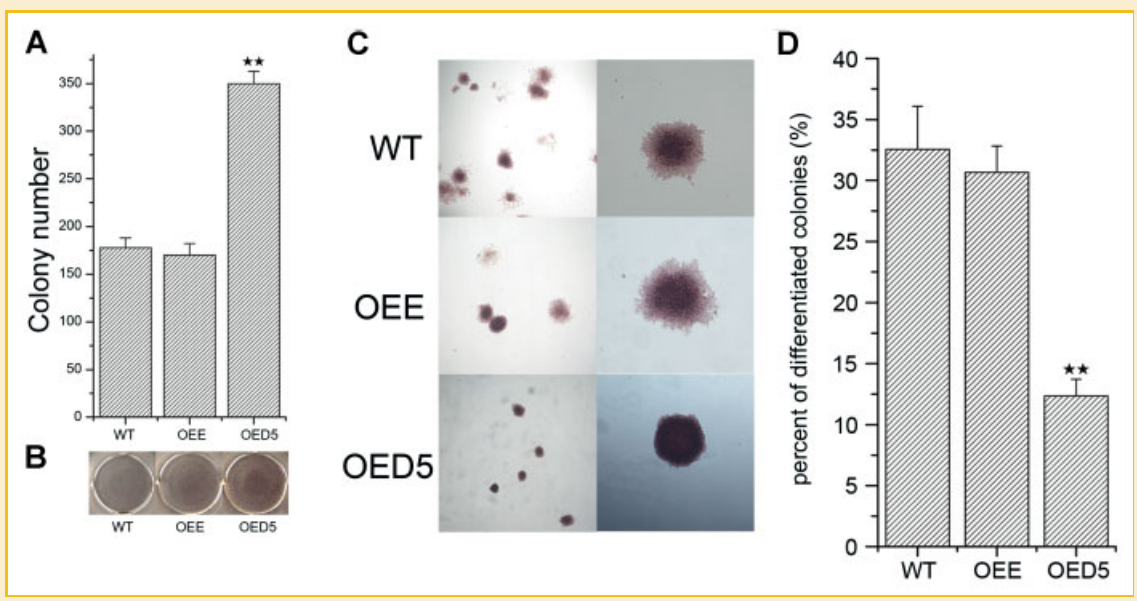

Fig. 2. Exogenous Dusp5 promoted the pluripotency maintenance ability of mES cells. A and B: Clonogenic assay of WT, OEE, and OED5 cells. Colony numbers were counted (A) and cultures were photographed (B). C and D: Colony differentiation assay of WT, OEE, and OED5 cells. Cultures were photographed (C) and differentiated colonies were counted (D). Error bar represents the mean values ± S.D. from four independent experiments. $^{**}P < 0.01$. WT, wild type mES D3 cells; OEE, mES D3 cells over-expressing the EGFP protein; OED5, mES D3 cells over-expressing the Dusp5 protein.

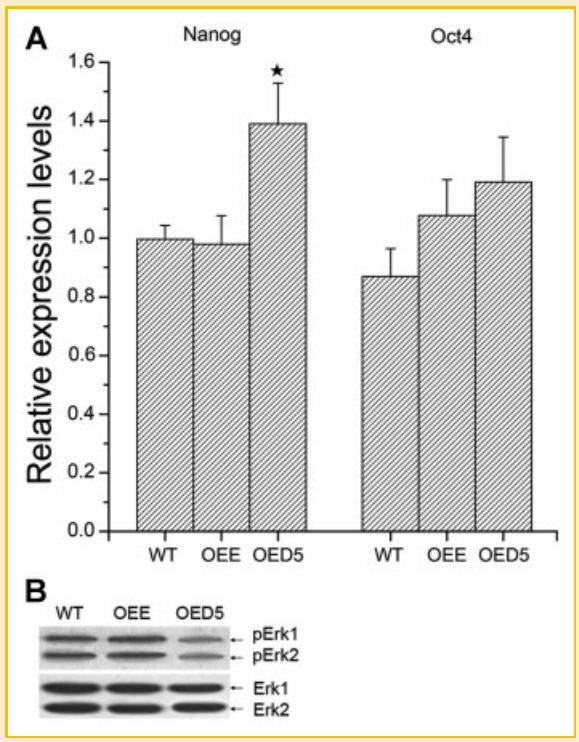

Fig. 3. Exogenous Dusp5 retained *Nanog* and *Oct4* expression in mES cells. A: *Nanog* and *Oct4* transcription levels were analyzed by qRT-PCR (n = 4) in mES cells. Gene expression was normalized against a β-actin internal control. Error bar represents the means ± S.D. from four independent experiments. $^{*}P < 0.05$. B: Western blot analysis of WT, OEE, and OED5 cells with an anti-Erk-1/2 antibody and an anti-phosphorylated Erk-1/2 antibody. Immunoblot signals are indicated by arrows. WT, wild type mES D3 cells; OEE, mES D3 cells over-expressing the EGFP protein; OED5, mES D3 cells over-expressing the Dusp5 protein.

To assess the effects of *Dusp5* knockdown on mES cell differentiation, ES cell markers were analyzed by qRT-PCR, which showed that *Nanog* and *Oct4* transcript levels in *Dusp5* knockdown cells decreased significantly (Fig. 4A and B). To determine whether Erk activity was up-regulated by *Dusp5* knockdown, these cells were analyzed by Western blot analysis with antibodies against phosphorylated and unphosphorylated Erk. *Dusp5* knockdown cells showed stronger Erk2 phosphorylation signals than the two controls, and a slight rise in Erk1 activity was also detected (Fig. 4C).

Clonogenic assays and colony differentiation assays were performed, and SHD5-pool, SHD5-g2, SHD5-e5, Scr, and WT cells were seeded and stained; however, no significant differences were observed between the cell lines (data not shown). The SHD5-g2 and SHD5-e5 cells were passaged for more than 20 generations without a notable decline in fluorescence or a change in morphology. In addition, it was observed that these *Dusp5* knockdown cells were capable of EB formation. It is worth noting that *Dusp5* knockdown did not affect the proliferation of mES cells.

DUSP5 KNOCKDOWN AFFECTS EB DEVELOPMENT BY DOWN-REGULATING GATA6, FLK1 AND TWIST1 TRANSCRIPTION

The effects of *Dusp5* knockdown were analyzed during EB development. After formation and culture, 4-day-old EBs were plated onto gelatin-coated plastic surfaces. EBs derived from WT (WTEBs) and from Scr cells (ScrEBs) attached and flattened rapidly, exhibiting flourishing cell growth, migration of the parietal endoderm and a collar of visceral endoderm cells at their margins. In contrast to these control EBs, EBs derived from *Dusp5* knockdown cells (KDEBs) showed weak adherence capability, very little outgrowth, and a lack of epithelial-like cells (Fig. 5A).

Next, the diameters of the EBs, including the outgrowth of endoderm cells when attached, were measured. Extremely abnormal

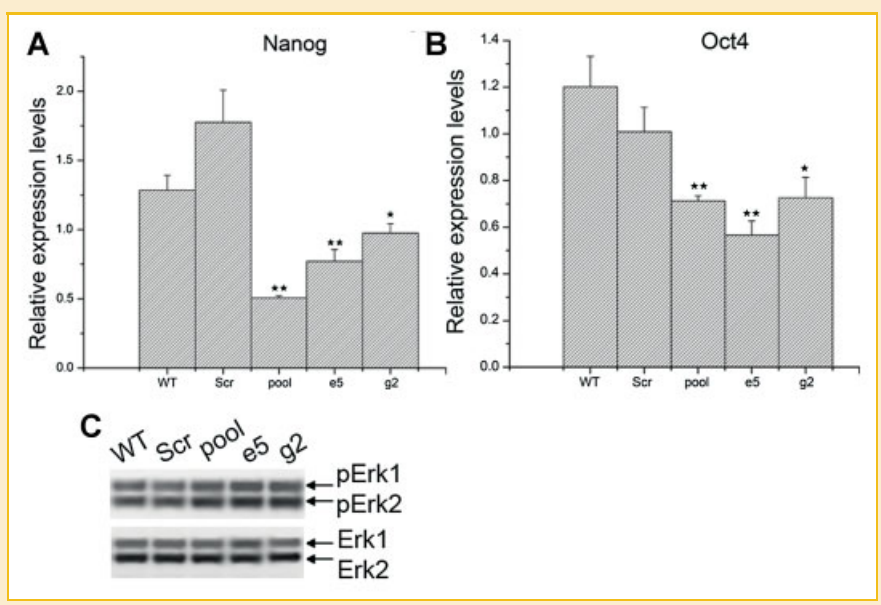

Fig. 4. Effects of *Dusp5* knockdown in mES cells. A and B: The transcription characteristics of *Nanog* (A) and *Oct4* (B) in mES cells were analyzed by qRT-PCR (n = 4). Gene expression was normalized against a β-actin internal control. Error bar represents the means ± S.D. from four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. C: Western blot analysis of mES cells with an anti-Erk-1/2 antibody and an anti-phosphorylated Erk-1/2 antibody. Immunoblot signals are indicated by arrows. WT, wild type mES D3 cells; Scr, scramble shRNA-treated mES D3 cells; pool, SHD5-pool cells; g2, SHD5-g2 cells; e5, SHD5-e5 cells.

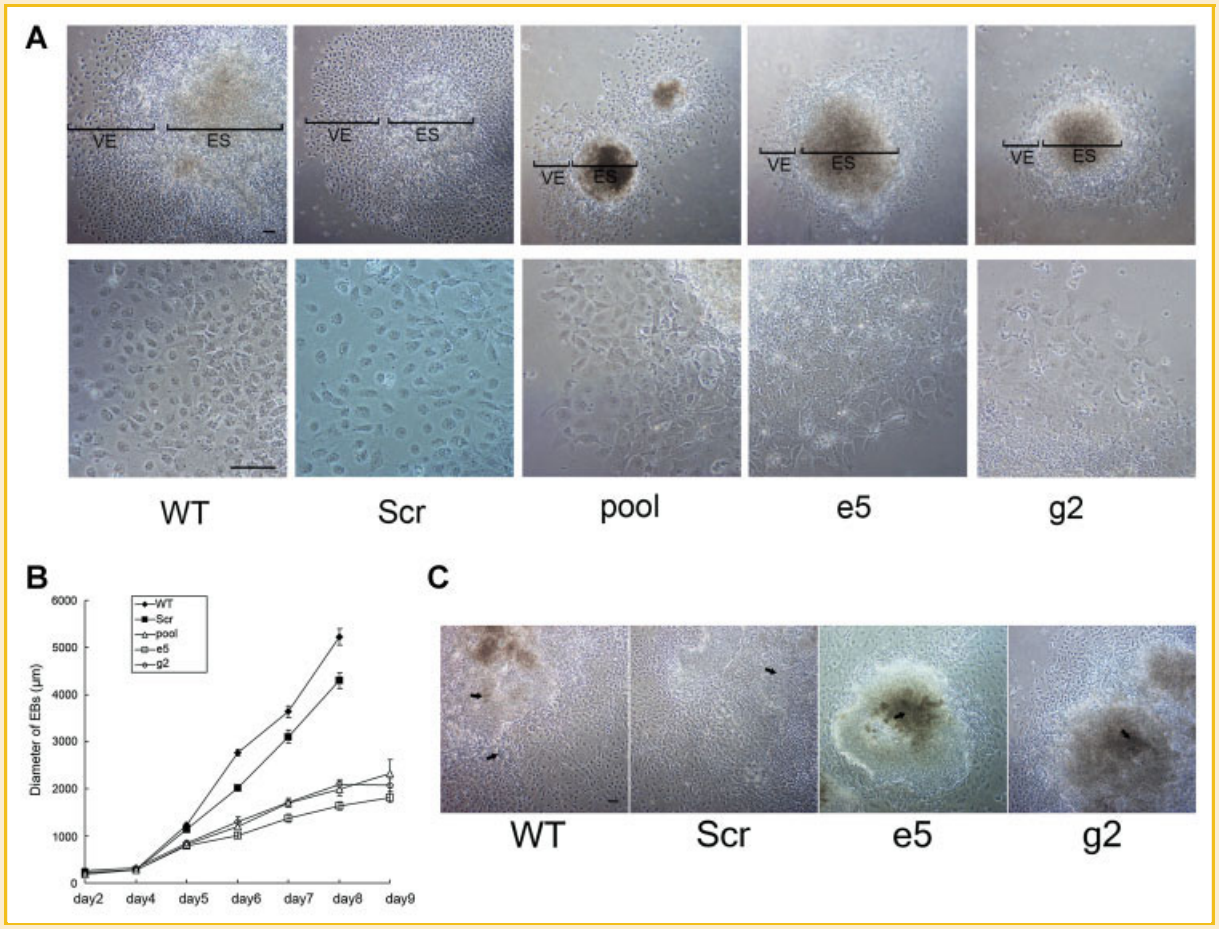

Fig. 5. *Dusp5* knockdown influences EB development. A: Adherent culture assay of EBs on day 6 (adhering day 2). Lines indicate visceral endoderm cells (VE) and undifferentiated mES cells (ES). B: EB diameters. After formation, EBs were cultured in suspension for 2 days and were then allowed to attach to a plastic surface. Photographs were taken under a phase contrast microscope everyday, and the EBs were measured (n = 10). C: Spontaneous cardiomyocyte differentiation of mES cells. Photographs were taken under a phase contrast microscope on day 12 (adhering day 7). Arrows indicate contracting aggregates. Bar = 100 μm. WT, wild type mES D3 cells; Scr, scramble shRNA-treated mES D3 cells; pool, SHD5-pool cells; g2, SHD5-g2 cells; e5, SHD5-e5 cells.

EBs were excluded from the results (Fig. 5B). On day 6 (adhering day 2), the sizes of KDEBs were only half that of EBs from the two control cell lines, and the gap widened steadily with time. Similar results were achieved even when the starting cell number for EB formation was varied from 15,000 to 60,000 cells/ml.

Spontaneously beating aggregates were observed initially in both attached KDEBs and control EBs on day 11. Following day 5, almost all beating cardiomyocytes were found in the outgrowths of attached WTEBs and ScrEBs as described previously [Takahashi et al., 2003; Koike et al., 2007]. In contrast, in KDEBs derived from SHD5-e5 and g2 cells, all contracting cells resided in the centers of the attached cells (Fig. 5C).

To define the mechanisms underlying the effects of *Dusp5* knockdown-mediated changes on EB differentiation, several specific germ layer marker genes including *Gata6*, *Gata4*, *Sox7*, *FoxA2*, and *Sox17* (endoderm), *brachyury T*, *Flk1*, and *Twist1* (mesoderm), and *Nestin*, *Fgf5I*, and *Sox1* (ectoderm) were examined quantitatively. As illustrated in Figure 6, a delay and decrease in the expression of *Gata6* in KDEBs, derived from SHD5-e5 and g2 cells, was observed. *Flk1* was expressed at extremely low levels, and the expression of *Twist1* was also suppressed in KDEBs. No significant differences were detected for the other genes tested.

Based on the abnormal morphology and reduced expression levels of the prominent germ layer markers, *Gata6*, *Flk1*, and *Twist1* in KDEBs, it was concluded that Dusp5 plays a critical role in the regulation of endoderm and mesoderm differentiation during EB development.

DISCUSSION

In recent years, ES cells have been defined as cells that capture the transient developmental phase of pluripotent blastocysts [Wray et al., 2010]. Since treatment with FGF4/Erk signaling inhibitors maintains the general stemness qualities of ES cells, MAPK/Erk signaling is believed to be at the center of pluripotency.

Recent reports and microarray data have implied that *Dusp6* and *Dusp7* are pluripotency-associated genes [Zhang et al., 2009; Abujarour et al., 2010], and both proteins inactivate their target kinase, Erk, by dephosphorylation [Muda et al., 1996; Dowd et al.,

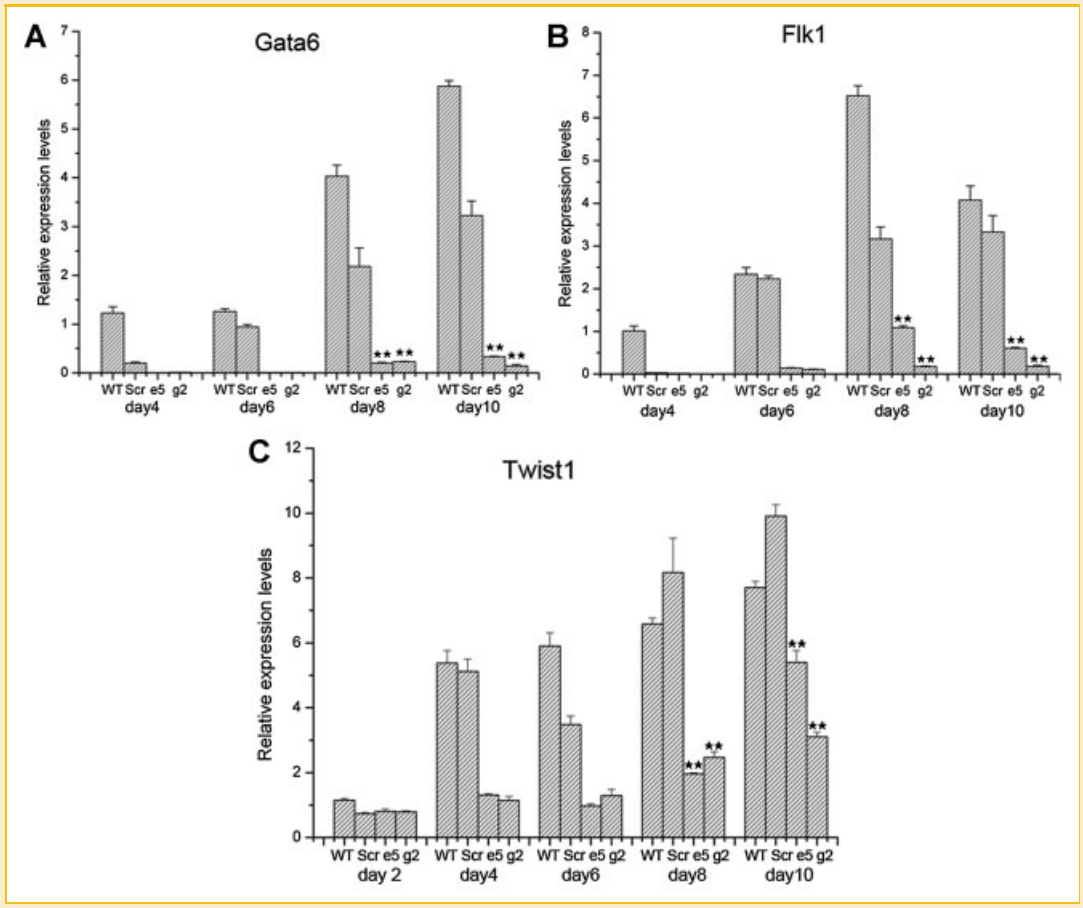

Fig. 6. *Gata6* (A), *Flk1* (B), and *Twist1* (C) mRNA expression in EB development was analyzed by qRT-PCR ($n = 4$). Gene expression was normalized against a β-actin internal control. Error bar represents the means ± S.D. from four independent experiments. $^{**}P < 0.01$. WT, wild type mES D3 cells; Scr, scramble shRNA-treated mES D3 cells; g2, SHD5-g2 cells; e5, SHD5-e5 cells.

1998]. This involvement of Dusp6 and Dusp7 in the negative regulation of Erk suggested that Dusp5 expression might also correlate with ES cell pluripotency. In the present study, we focused on Dusp5, which specifically suppresses the Erk cascade. *Dusp5* transcription was reduced dramatically during the early stages of EB formation and monolayer differentiation, and then gradually increased during further differentiation. Our data linked Dusp5 to the regulation of the pluripotent stage and differentiation.

Over the last few years, many reports have shown that suppression of Erk signaling promotes ES cell pluripotency [Burdon et al., 1999; Burdon et al., 2002; James et al., 2003; Ying et al., 2008; Nichols et al., 2009]. FGF, an important upstream activating signal in the Erk pathway, has been reported to be a crucial controller of pluripotency and lineage specification [Kunath et al., 2007; Lanner and Rossant, 2010]. Microarray assays showed that inhibition of FGF signaling down-regulated DUSP4/6 expression [Lanner et al., 2010]. Interestingly, our qRT-PCR analysis of *Dusp5* expression demonstrated that the addition of bFGF to the culture medium rapidly induced *Dusp5* expression in mES cells (Fig. S3). The increase in *Dusp5* expression in response to bFGF implies that Dusp5 is part of a negative feedback loop in FGF signaling, as reported previously in *Xenopus* embryos [Branney et al., 2009].

In our study, OED5 cells over-expressing Dusp5 were established. Clonogenic assays and colony differentiation assays showed that exogenous Dusp5 approximately doubled the ability of mES cells to maintain pluripotency. OED5 cells showed significantly higher levels of *Nanog* expression and a trend towards the up-regulation of *Oct4* expression when the LIF concentration was reduced by half. These results indicated that Dusp5 suppressed mES cell differentiation, by maintaining *Nanog* and perhaps *Oct4* expression, and by inhibiting Erk activity. On the other hand, the *Dusp5* knockdown mES cells revealed a significant down-regulation of *Nanog* and *Oct4* expression, and an increase in Erk activity.

From the findings in this study, we conclude that Dusp5 affects pluripotency by regulating *Nanog* and *Oct4* expression, possibly via the FGF/Erk signaling pathway. When cultured in the absence of anti-differentiation factors such as LIF, mES cells spontaneously differentiated and Dusp5 expression was down-regulated, while exogenous Dusp5 rescued mES cell pluripotency. Dusp5, a short-lived protein, is considered to act as a negative feedback regulator of stimulated Erk signaling in somatic cells [Kucharska et al., 2009]. We believe that high basal expression of Dusp5 is required for the arrest of differentiation when Erk signaling is activated during early inner cell mass expansion (Fig. S4).

Although *Dusp5* knockdown mES cells were capable of forming EBs, they exhibited some unusual characteristics, for example, they grew slowly, showed very little outgrowth after adhering to gelatin-coated tissue culture plates, and were only half of the size of the controls. In addition, *Gata6*, *Flk1*, and *Twist1* gene expression was suppressed significantly during EB development following *Dusp5* knockdown. These results suggested that Dusp5 is required for endoderm and mesoderm differentiation during EB development (Fig. S4). It was reported that *Gata6* knockout embryos fail to form functional visceral endoderm, and widespread, programmed cell death has been observed within the embryonic ectoderm of *Gata6*-deficient embryos [Morrisey et al., 1998; Koutsourakis et al., 1999]. Moreover, the loss of *Gata6* leads to a lack of epithelial differentiation and neonatal cell death [Zhang et al., 2008]. In zebrafish embryos, Dusp5 has been shown to regulate *Flk1* expression during angioblast development in vivo, while *Dusp5* knockdown causes apoptosis of endothelial cells in vitro [Pramanik et al., 2009]. We believe that the decrease in the expression of *Gata6* and *Flk1* may account for the apoptosis of outgrowth cells in KDEBs, following *Dusp5* knockdown. Dusp5 is part of a negative feedback loop in FGF signaling, as reported previously [Branney et al., 2009; Lanner and Rossant 2010], and our results suggested that *Gata6* regulation downstream of the FGF/Ras/Erk signal pathway must be precisely controlled [Li et al., 2004; Chazaud et al., 2006; Yamanaka et al., 2010]. Over-activation of Erk may block transcription of *Gata6* in EB development.

Cardiomyocyte differentiation of KDEBs was also unusual in that all spontaneous beating cardiomyocytes appeared in the center of the KDEBs rather than in the outgrowths. These results indicated that Dusp5 might not be essential for cardiomyocyte differentiation, but instead might play a role in the migration of cardiomyocytes in EBs. Twist1, a basic helix–loop–helix transcription factor, is an important developmental control gene in mesoderm development [Füchtbauer, 1995; Stoetzel et al., 1995], and has been reported to be essential for cardiac cushion epithelial–mesenchymal transition during embryogenesis [Ma et al., 2005; Shelton and Yutzey, 2008]. In addition, studies have shown that Flk1 regulates cell migration during early development [Shalaby et al., 1997]; thus, it is likely that reduced *Flk1* and *Twist1* expression explains the failure of the beating cardiomyocyte aggregates to migrate from the center of the KDEBs.

In summary, this study has demonstrated the pluripotency-associated expression and pluripotency maintenance ability of Dusp5 in mES cells. Additionally, our results revealed that Dusp5 is required for EB development, possibly via regulation of the FGF/Erk signaling pathway. Additional studies will be needed to determine the exact molecular mechanism involved in the precise control of Erk activation by Dusp5 during embryogenesis.

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