

In vitro chondrogenesis of hSSCs and transduction with lentivirus vectors

hSSCs were isolated from the synovium of the human adult knee joint during arthroscopic surgery in accordance with a protocol approved by the Osaka University Institutional Ethics Committee (Tateishi *et al.* 2007). They were then cultured in high-glucose Dulbecco's modified Eagle's medium (HG-DMEM; Gibco BRL) containing 10% fetal bovine serum and 1% penicillin/streptomycin. The cDNA encoding Akt-Mer was inserted into lentivirus vector pWPI. Subconfluent 293T cells were co-transfected with pWPI-Akt-Mer, pCMV-AR8.91, and pMD2G-VSVG using FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 h, the medium was changed, and recombinant lentivirus was harvested 36 h later (Wiznerowicz & Trono 2003). The hSSCs of passage three were infected with the lentivirus, and the cells of passage five were used for chondrogenic differentiation. A total of 2×10^5 cells were placed in a 15-mL polypropylene tube and centrifuged at 500 g for 5 min. The pellets were cultured for 14 or 21 days in chondrogenic medium of HG-DMEM supplemented with 10 ng/mL TGF β 1 (R&D Systems, Minneapolis, MN), 50 μ g/mL ascorbic acid, 100 μ g/mL sodium pyruvate, 100 nM dexamethasone, and 50 mg/mL each of insulin, transferrin, and selenious acid (ITS Premix; BD Biosciences, San Jose, CA). The agents 4OHT, LY294002, and NL71-101 (Calbiochem) were used at 1 μ M. The cross-sectional area of the pellets was measured using Leica IM500 software.

Histological analysis

Cultured limb explants and pellets were fixed overnight in 4% paraformaldehyde at 4 °C, embedded in methyl methacrylate, and cut into 5- μ m sections. The sections were stained for 2 h in 1% Alcian blue (Alcian Blue 8GX; Sigma) and subsequently counterstained with Kernechtrot.

Quantification of GAG levels

The pellets were digested for 4 h at 65 °C with a Papain solution (Sigma) and assayed for GAG as a measurement of proteoglycan content. GAG levels were measured using the 1,9-dimethylmethylene blue binding assay on the basis of a chondroitin sulfate standard curve (Nacalai Tesque, Kyoto, Japan) (Karran *et al.* 1995; Tateishi *et al.* 2007).

Antibodies

Antibodies against phospho (Ser473)-Akt, and type X collagen were purchased from Cell Signaling Technology (Danvers, MA), and LSL (Cosmo Bio, Tokyo, Japan), respectively.

In situ hybridization

Digoxigenin-11-UTP-labeled single-stranded RNA probes were prepared using a DIG RNA Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufac-

turer's instructions. Hybridization was performed as described previously (Hirakawa *et al.* 1994; Kawahata *et al.* 2003).

Semi-quantitative reverse transcription PCR analysis

Total RNA was extracted using Sepasol(R)-RNA 1 Super (Nacalai Tesque), and 1 μ g of total RNA was used for cDNA synthesis. First-strand cDNA was synthesized using the ThermoScript RT-PCR System (Gibco BRL). PCR reactions were optimized to allow semi-quantitative comparisons within the log phase of amplification. The primer sequences and amplification cycles are listed in Supplementary Table S1 (Giotto *et al.* 2003; Ikeda *et al.* 2005). The PCR products were analyzed by agarose gel electrophoresis and visualized using ethidium-bromide staining.

Acknowledgement

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the 21st Century COE "CICET".

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Received: 28 January 2008

Accepted: 11 May 2008

Supplementary material

The following supplementary materials are available for this article online:

Figure S1 Effects of Akt inhibition on chondrocyte differentiation in hSSCs.

Table S1 Primers used for RT-PCR amplification

This material is available as part of the online article from:

<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2443.2008.01209.x>

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AKT signaling promotes derivation of embryonic germ cells from primordial germ cells

Tohru Kimura¹, Maya Tomooka², Noriko Yamano², Kazushige Murayama¹, Shogo Matoba³, Hiroki Umehara², Yoshiakira Kanai³ and Toru Nakano^{1,2,*}

Primordial germ cells (PGCs) are embryonic germ cell precursors. Although the developmental potency of PGCs is restricted to the germ lineage, PGCs can acquire pluripotency, as verified by the *in vitro* establishment of embryonic germ (EG) cells and the *in vivo* production of testicular teratomas. PGC-specific inactivation of PTEN, which is a lipid phosphatase antagonizing phosphoinositide-3 kinase (PI3K), enhances both EG cell production and testicular teratoma formation. Here, we analyzed the effect of the serine/threonine kinase AKT, one of the major downstream effectors of PI3K, on the developmental potency of PGCs. We used transgenic mice that expressed an AKT-MER fusion protein, the kinase activity of which could be regulated by the ligand of modified estrogen receptor (MER), 4-hydroxytamoxifen. We found that hyperactivation of AKT signaling in PGCs at the proliferative phase dramatically augmented the efficiency of EG cell establishment. Furthermore, AKT signaling activation substituted to some extent for the effects of bFGF, an essential growth factor for EG cell establishment. By contrast, AKT activation had no effect on germ cells that were in mitotic arrest or that began meiosis at a later embryonic stage. In the transgenic PGCs, AKT activation induced phosphorylation of GSK3, which inhibits its kinase activity; enhanced the stability and nuclear localization of MDM2; and suppressed p53 phosphorylation, which is required for its activation. The p53 deficiency, but not GSK3 inhibition, recapitulated the effects of AKT hyperactivation on EG cell derivation, suggesting that p53 is one of the crucial downstream targets of the PI3K/AKT signal and that GSK3 is not.

KEY WORDS: AKT, Primordial germ cells, EG cells, Pluripotency, Stem cells, Mouse

INTRODUCTION

Primordial germ cells (PGCs) are embryonic germ cell precursors that differentiate into spermatozoa and oocytes (McLaren, 2003; Wylie, 2000). PGCs emerge from precursor cells in the proximal epiblast at embryonic day 7.25 (E7.25) in mice. PGCs migrate through the hindgut and dorsal mesentery to colonize the genital ridges by E11.5. The PGCs proliferate from about 45 founder cells to 25,000 cells between E7.25 and E13.5. In males, the cells enter mitotic arrest at E13.5, and spermatogonia resume proliferation and undergo spermatogenesis after birth. By contrast, female PGCs enter meiosis at E13.5, and oocytes mature to ovulation after birth.

Although the developmental potency of PGCs is restricted to the germ lineage in normal development, two lines of evidence suggest that mammalian PGCs can dedifferentiate into cells with broader differentiation potential (Kimura et al., 2005). First, when PGCs are cultured in the presence of leukemia inhibitory factor (LIF), stem cell factor (SCF) and basic fibroblast growth factor (bFGF), they give rise to embryonic germ (EG) cells (Matsui et al., 1992; Resnick et al., 1992). EG cells possess pluripotency similar to that of embryonic stem (ES) cells, as EG cells contribute to the somatic and germ lineages after being introduced into blastocysts (Labosky et al., 1994; Stewart et al., 1994). However, freshly isolated PGCs do not contribute to any tissues upon transfer into blastocysts, which indicates that some reprogramming events are necessary for the development of EG

cells from PGCs (Durova-Hills et al., 2006). Second, germ cell tumors, called teratocarcinomas, contain a range of differentiated cell types, including more than two germ layers and a population of undifferentiated embryonic cells, known as embryonal carcinoma (EC) cells. The tumors have been shown experimentally to originate from PGCs (Stevens, 1967).

Phosphoinositide 3-kinase (PI3K), which is activated by a number of growth factors, cell adhesion molecules and chemokines, produces the second messenger molecule phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P₃) from PtdIns(4,5)P₂ (Cantley, 2002). PtdIns(3,4,5)P₃ then transmits the signal via various downstream effectors, including the serine/threonine kinase AKT, and regulates proliferation, survival, migration, metabolism and tumorigenesis (Brazil et al., 2004). By contrast, the tumor suppressor PTEN dephosphorylates PtdIns(3,4,5)P₃ to PtdIns(4,5)P₂, thereby antagonizing the physiological and pathological actions of PI3K signaling (Kishimoto et al., 2003; Stiles et al., 2004). An analysis of PGC-specific *Pten*-deficient mice has shown that PI3K signaling promotes the dedifferentiation of PGCs (Kimura et al., 2003; Moe-Behrens et al., 2003). In particular, the deletion of *Pten* increased the cloning efficiency of EG cells and led inevitably to the development of testicular teratomas. PI3K signaling exerts its biological effects through the activation of various downstream molecules, which include AKT and the GTPases RAC and CDC42 (Brazil et al., 2004; Cantley, 2002). It remains to be elucidated which PI3K downstream molecules mediate the effects of *Pten* deletion in the dedifferentiation of PGCs.

AKT is hyperphosphorylated in PGCs and early teratomatous foci in PGC-specific *Pten*-deficient mice (Kimura et al., 2003), and PI3K/AKT signaling plays an important role in the regulation of ES cell pluripotency (Ivanova et al., 2006; Paling et al., 2004; Watanabe et al., 2006). We examined the effect of activating AKT signaling on EG cell derivation and found that AKT activation

¹Department of Pathology, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. ²Graduate School of Frontier Biosciences, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. ³Department of Veterinary Anatomy, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan.

*Author for correspondence (e-mail: tnakano@patho.med.osaka-u.ac.jp)

considerably increased EG cell formation from E11.5 PGCs in the presence of LIF, SCF and bFGF, as had been seen for the *Pten*-deficient PGCs. In addition, AKT activation efficiently promoted EG cell derivation, even in the absence of bFGF. However, the signal did not promote the establishment of EG cells from germ cells after E15.5, by which time mitotic arrest and meiosis had occurred in the male and female germ cells, respectively. Our study indicates that the PI3K/AKT signaling axis controls the derivation of EG cells from PGCs.

MATERIALS AND METHODS

Mice

To create β -actin promoter-driven AKT-MER transgenic mice, *Akt-Mer* cDNA was subcloned into the *EcoRI* site of the pCAGGS-IRES-EGFP vector by blunt-ended ligation (Watanabe et al., 2006). The prokaryotic sequences were excised from the plasmid, and the gel-isolated DNA fragment was injected into the pronuclei of BDF1 fertilized zygotes. Founder mice were screened by PCR on tail DNA and were bred in the BDF1 background (Murayama et al., 2007). The morning on which a copulation plug was noted was defined as embryonic day 0.5 (E0.5). The p53-deficient mice with mixed background (C57BL/6, CBA2 and ICR) were described previously (Tsukada et al., 1993). Animal care procedures were in accordance with the guidelines of Osaka University.

PGC culture

Gonads were dissociated into single cells by incubation in 0.05% trypsin and 0.02% EDTA in PBS for 10 minutes. Dispersed suspensions of germ cell-containing tissues were cultured on SI/SI⁴-m220 feeder cells in 24-well plates with DMEM that was supplemented with 15% KNOCKOUT Serum Replacement (Invitrogen, Carlsbad, CA), 2 mM glutamine, 1 mM sodium pyruvate, and nonessential amino acids, in the presence or

absence of 1000 U/ml LIF, 20 ng/ml bFGF (R&D Systems, Minneapolis, MN), and 10 μ M forskolin (Sigma-Aldrich, St Louis, MO) (Koshimizu et al., 1996; Matsui et al., 1992). The primary cultures were passaged to SI/SI⁴-m220 feeder cells (secondary cultures) and subsequently to mouse embryonic fibroblasts (MEFs; tertiary cultures) every 5 days (see Fig. 2A). For the secondary and tertiary cultures, 4OHT and bFGF were omitted from the medium. When PGCs were cultured with LIF alone, the cell suspensions were seeded onto MEFs in six-well plates and passaged to MEFs until tertiary culture.

The SI/SI⁴-m220 cells were treated with 5 μ g/ml mitomycin C for 1 hour and plated at 4×10^5 cells/well in 24-well plates one day before use. The MEFs were treated with 10 μ g/ml mitomycin C for 2 hours and plated at 1.1×10^5 cells/well in 24-well plates or at 6.3×10^5 cells/well in six-well plates. PGCs and EG cells were fixed using 4% paraformaldehyde (PFA) and visualized using an alkaline phosphatase staining kit (Sigma). The number of adherent PGCs at 8 hours post-seeding was defined as the number of seeded PGCs. Multilayered colonies with more than 20 cells at day 5 of primary culture were considered to be primary EG cell colonies, as described (Kimura et al., 2003; Koshimizu et al., 1996; Moe-Behrens et al., 2003).

Immunostaining and flow cytometry

PGC cultures were fixed with 4% PFA and stained as described (Kimura et al., 2006). The primary antibodies used were anti-Ser473-phosphorylated AKT antibody (1:50 dilution; Cell Signaling Technology, Beverly, MA), anti-phospho-GSK3 α/β antibody (1:50; Cell Signaling), anti-Ser20-phospho-p53 antibody (1:25; Cell Signaling), MDM2 (1:50; Santa Cruz, CA, USA), PGC7/Stella (1:1000; DPPA3 – Mouse Genome Informatics) (Sato et al., 2002), and SSEA-1 (1:50; Kyowa Hakko, Tokyo, Japan; FUT4 – Mouse Genome Informatics). Genital ridge suspensions were stained with the SSEA-1 antibody and were analyzed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ).

Fig. 1. PGCs of AKT-MER transgenic mice.

(A) AKT-MER transgenic mice (right) and littermate control mice (left) at E11.5. As the *Akt-Mer* cDNA is linked to *IRES-EGFP*, the transgenic mice can be identified by EGFP fluorescence. **(B)** Flow cytometry analysis of cells from the gonads of E11.5 AKT-MER mice. Suspensions were stained for the PGC-specific marker SSEA-1. EGFP fluorescence was detected in the majority of the transgenic PGCs. **(C)** Level of AKT activation in the cultured PGCs. The E11.5 PGCs were seeded onto SCF-expressing SI/SI⁴-m220 feeder cells and cultured with or without bFGF and 4OHT for 1 day. The cells were stained with the SSEA-1 (green) and anti-Ser473-phosphorylated-AKT (pAKT) (red) antibodies. The fluorescence intensity of phospho-AKT in the individual PGCs was measured using LSM5 PASCAL confocal microscopy. Relative fluorescence per cell is shown in the right panel (mean \pm s.e.m.). The background fluorescence level was measured in the samples that were not treated with pAKT antibody. Phospho-AKT signals were stronger in the 4OHT-treated transgenic PGCs than in the untreated transgenic and the bFGF-treated wild-type controls (* $P < 0.01$, # $P < 0.05$, Student's *t*-test). The signals were stronger in the transgenic PGCs than in the control PGCs, even in the absence of 4OHT and bFGF (* $P < 0.05$).

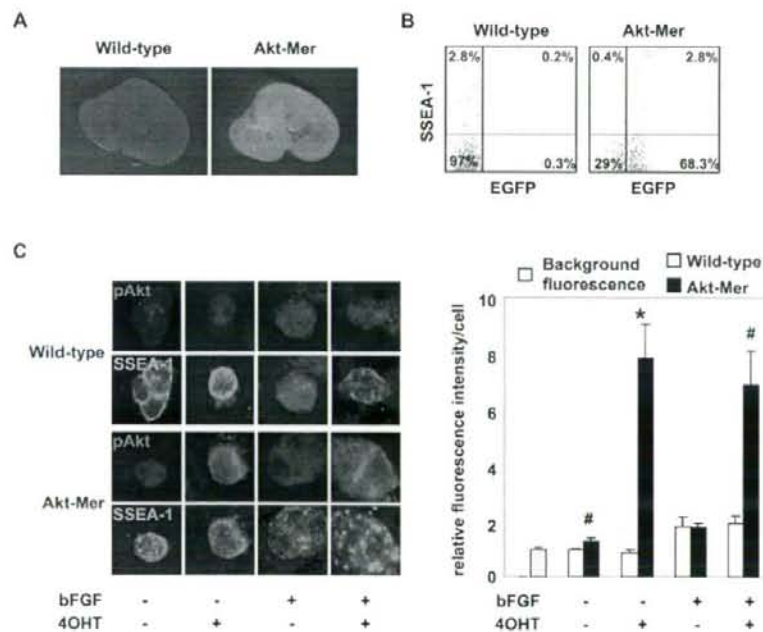
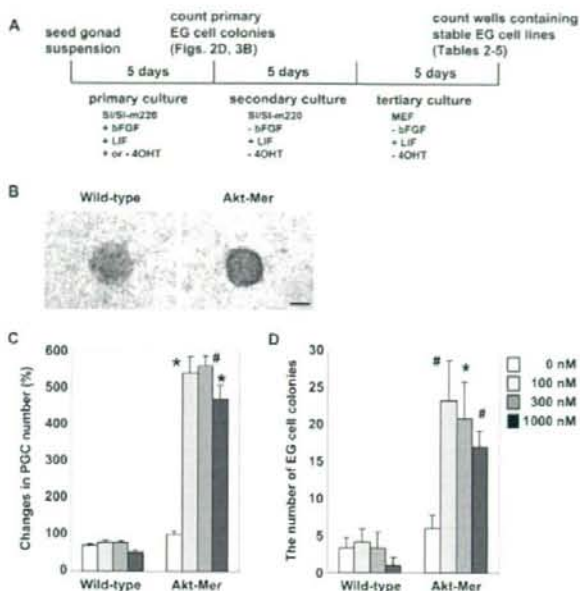


Fig. 2. EG cell production from AKT-MER-expressing PGCs under standard conditions. (A) Procedure for EG cell derivation. Cell suspensions of E11.5 gonads were seeded onto *Sl/Si^h-m220* feeder cells that express the membrane-bound form of SCF, and cultured with LIF and bFGF (primary culture). 4OHT and bFGF were added only during primary culture. At day 5 of primary culture, whole wells were trypsinized and passaged onto fresh *Sl/Si^h-m220* layers. Secondary culture was carried out with LIF for 5 days. The whole wells were finally passaged onto MEFs and cultured with LIF (tertiary culture). At day 5 of tertiary culture, the wells containing EG cells were counted to determine the derivation efficiency. The EG cells at tertiary culture were propagated to stable cell lines on MEFs in the presence of LIF. A fraction of EG cells in the primary cultures (primary EG cells) gave rise to stable EG cell lines by this whole-well passage procedure. The efficiency is detailed in the legend of Table 2A.

(B) Alkaline phosphatase staining of primary EG cell colonies derived from wild-type PGCs cultured without 4OHT (left) and of transgenic PGCs cultured with 100 nM 4OHT (right). The EG cell colonies were multilayered and positive for alkaline phosphatase activity. Scale bar, 100 μ m. (C) Increased number of AKT-MER-expressing PGCs. The cells of 0.02 embryos were added to each well, and 30–40 PGCs attached to feeder cells. The percentage change in the PGC population was calculated by dividing the number of PGCs at day 3 by the number of attached PGCs. The 4OHT-treated transgenic PGCs proliferated significantly more than untreated transgenic PGCs (mean \pm s.e.m.; * P <0.005, # P <0.0005, Student's *t*-test; n =6) and wild-type PGCs cultured with the corresponding concentrations of 4OHT (P <0.005, P <0.0005 and P <0.005 for 100 nM, 300 nM and 1000 nM, respectively). (D) Increased primary EG cell colony formation by E11.5 AKT-MER-expressing PGCs. A primary EG cell colony was defined as a multilayered, alkaline phosphatase-positive colony of more than 20 cells, as described previously (Kimura et al., 2003; Moe-Behrens et al., 2003). Colony formation was significantly higher for 4OHT-treated transgenic PGCs than for untreated transgenic PGCs (mean \pm s.e.m.; * P <0.005, # P <0.0005, Student's *t*-test; n =6) and wild-type PGCs cultured with the corresponding concentrations of 4OHT (P <0.0005, P <0.005 and P <0.0005 for 100 nM, 300 nM and 1000 nM, respectively).



Apoptosis and proliferation index

Apoptotic cells were stained with fluorescently labeled caspase inhibitor (SR-VAD-FMK; BIOMOL, Plymouth Meeting, PA), which specifically binds to activated caspases, for 1 hour before fixation. PGCs were detected by immunostaining with SSEA-1. Mitotic cells were visualized by staining chromosomal DNA with DAPI (4',6-diamidino-2-phenylindole).

Differentiation assays

The *in vitro* induction of differentiation to hematopoietic cells from EG cells was performed as described (Nakano et al., 1994). For the teratoma formation assay, 5×10^5 EG cells were injected subcutaneously into nude mice (Japan SLC, Hamamatsu, Japan). After 3 weeks, the tumors were fixed with 4% PFA and embedded in OCT compound (Sakura Finetek, Tokyo). Frozen sections were cut and stained with Hematoxylin and Eosin. EG cells

Table 1. Apoptosis and mitosis in the cultured PGCs

A Apoptosis					
Treatment		% Apoptosis		χ^2 test	
bFGF	4OHT	Wild type	AKT-MER	-4OHT	Wild type
-	-	8.1% (25/309)	8.2% (30/365)		>0.05
-	+	6.3% (22/348)	2.0% (8/395)	<0.005	<0.005
+	-	2.2% (8/369)	3.0% (10/337)		>0.05
+	+	2.2% (8/362)	0.33% (1/302)	<0.025	<0.05

B Mitosis					
Treatment		% Mitosis		χ^2 test	
bFGF	4OHT	Wild type	AKT-MER	-4OHT	Wild type
-	-	0% (0/309)	0% (0/365)		n.a.
-	+	0% (0/348)	1.0% (4/395)	>0.05	>0.05
+	-	0.84% (3/369)	0.93% (3/337)		>0.05
+	+	0.85% (3/362)	4.2% (12/302)	<0.01	<0.01

PGCs of E11.5 embryos were cultured for 2 days under the indicated conditions. The apoptotic cells were labeled for 1 hour with SR-VAD-FMK before fixation. The PGCs were visualized with SSEA-1 staining, and mitotic cells were identified with DAPI staining.

n.a., not applicable.

Table 2. Effects of AKT activation on EG cell derivation from E11.5 and E8.5 PGCs

A							
Growth factors	Seeded PGC (embryos/well)	4OHT (nM)	EG cell-positive wells		χ^2 test		
			Wild type	AKT-MER	Wild type	0 nM	
bFGF, SCF, LIF	0.02	0	5/12	6/12	nsd	–	
	0.02	100	6/12	12/12	<0.005	<0.005	
	0.02	300	4/12	12/12	<0.005	<0.005	
	0.02	1000	3/12	12/12	<0.005	<0.005	
	0.02	100	7/12	12/12	<0.025	–	
	0.01	100	5/12	12/12	<0.005	–	
	0.005	100	3/12	11/12	<0.005	–	
	0.0025	100	2/12	8/12	<0.025	–	
	SCF, LIF	0.1	0	0/19	6/29	<0.05	–
		0.1	100	0/20	27/28	<0.005	<0.005
0.1		300	0/19	29/29	<0.005	<0.005	
0.1		1000	0/20	28/28	<0.025	<0.005	
0.02		0	0/12	1/12	nsd	–	
LIF	0.02	300	0/12	6/12	<0.005	<0.05	
	0.4	0	0/5	0/5	nsd	–	
	0.4	100	0/5	0/5	nsd	nsd	
	0.4	300	0/7	3/9	nsd	nsd	

B							
Growth factors	Seeded PGC (embryos/well)	4OHT (nM)	EG cell-positive wells		χ^2 test		
			Wild type	AKT-MER	Wild type	0 nM	
bFGF, SCF, LIF	0.1	0	4/15	4/10	nsd	–	
	0.1	100	5/15	10/10	<0.025	–	
SCF, LIF	0.5	0	0/7	0/6	<0.05	–	
	0.5	300	0/7	5/6	<0.005	<0.05	

(A) Cell suspensions from the genital ridges of E11.5 embryos were seeded onto SCF-expressing *Sl/Si⁴-m220* feeder cells at a density of 0.0025–0.1 embryos per well. The cells were cultured with bFGF and LIF (upper row), or with LIF alone (middle row), in the presence of various concentrations of 4OHT. Primary cultures were passaged to tertiary cultures to establish EG cell lines; secondary and tertiary cultures did not contain 4OHT or bFGF. The number of wells with EG cell colonies in the tertiary cultures, per total number of wells, is shown. In the experiments shown in the bottom row, cell suspensions were seeded onto MEFs from primary cultures at 0.4 embryos/well and cultured in the presence of LIF alone. Taken together with the results of primary cultures (Fig. 2D and Fig. 3B), we estimated the efficiency for establishing stable EG cell lines from primary colonies: 10–13% of wild-type primary EG cell colonies gave rise to stable cell lines, irrespective of 4OHT treatment. In the bFGF-treated transgenic cultures, 8.3% and 23% of primary colonies produced cell lines in the absence and presence of 100 nM 4OHT, respectively. In the absence of bFGF, 15% of the 300 nM 4OHT-treated primary colonies gave rise to cell lines.

(B) The caudal region of E8.5 embryos was seeded onto *Sl/Si4-m220* feeder layers and cultured with bFGF and LIF, or with LIF alone. 4OHT was added at the indicated concentration during primary culture.

nsd, not statistically different.

established by AKT activation were injected into blastocyst-stage embryos from C57BL/6 strain mice. The incorporation of cells into the chimeric mice was monitored by EGF fluorescence.

RESULTS

Production and characteristics of AKT-MER transgenic mice

To control AKT signaling activation in a conditional manner, we used the AKT-MER fusion protein, which is composed of a myristoylated, constitutively active form of AKT and the ligand-binding domain of mutant estrogen receptor (MER) (Kohn et al., 1998). The kinase activity of AKT-MER can be regulated by the addition or removal of the synthetic ligand of MER, 4-hydroxytamoxifen (4OHT). The fusion protein is in an inactive form in the absence of 4OHT but is activated rapidly by the addition of 4OHT (Kohn et al., 1998; Watanabe et al., 2006).

We used a cytomegalovirus enhancer linked to the β -actin promoter to drive the expression of AKT-MER. Because *Akt-Mer* cDNA fuses to *IRE5-EGFP* (internal ribosomal entry site linked to enhanced green fluorescent protein), the transgenic mice can be identified by EGFP fluorescence (Fig. 1A). Flow cytometry analysis of the E11.5 gonad suspensions revealed that the majority of the SSEA-1-positive PGCs were positive for EGFP, showing that AKT-MER was efficiently expressed in the PGCs (Fig. 1B).

The level of AKT activation in the cultured PGCs was analyzed by immunostaining using a phospho-AKT-specific antibody (Fig. 1C). When wild-type PGCs were cultured without bFGF, the AKT phosphorylation level was comparable to background fluorescence level. However, phosphorylation was induced by bFGF treatment. In the transgenic PGCs, the addition of 4OHT induced a much stronger signal, regardless of bFGF treatment. A weak but significant phospho-AKT signal was also detected in the transgenic PGCs in the absence of 4OHT, suggesting leakiness of the kinase activity of the AKT-MER fusion protein without 4OHT.

Increased derivation of EG cells by AKT signaling activation

We cultured PGCs from E11.5 embryos on SCF-expressing *Sl/Si⁴-m220* cells in the presence of LIF and bFGF, which is the standard culture procedure for establishing EG cell lines (Fig. 2A) (Matsui et al., 1992; Resnick et al., 1992). We used a mixture of gonads from male and female mice, as the proliferation rates and EG cell-forming efficiencies of the PGCs from the two sexes at E11.5 were equal and the *Pten* deletion enhanced PGC proliferation and EG cell production regardless of sex (Kimura et al., 2003). Although E11.5 PGCs rapidly undergo apoptosis in vitro, SCF, LIF and bFGF act as survival and growth factors (De Felici and Dolci, 1991; Godin et al., 1991; Koshimizu et al., 1995; Matsui et al., 1991). When seeded onto the feeder layer, morphology and adhesion were not altered by

Table 3. Effects of PI3K inhibition and AKT activation on EG cell derivation

Treatment		Seeded PGC (embryo/well)	EG cell-positive wells	
4OHT	LY294002		Wild type	AKT-MER
-	-	0.02	7/12	6/12
-	+	0.02	0/12	0/12
+	-	0.02	6/12	12/12
+	+	0.02	0/12	12/12
+	-	0.005	nd	7/12
+	+	0.005	nd	9/12
+	-	0.0025	nd	5/12
+	+	0.0025	nd	4/12

Cell suspensions from the genital ridges of E11.5 embryos were seeded onto SV/Sl⁴-m220 feeder cells at a density of 0.0025-0.02 embryos per well. The cells were cultured with bFGF and LIF in the presence of 1 mM 4OHT and/or 10 μ M LY294002. nd, not done.

AKT hyperactivation (data not shown). We then examined the rates of apoptosis and mitosis by staining with the fluorescently labeled caspase inhibitor SR-VAD-FMK and with DAPI, respectively. Apoptotic PGCs significantly decreased but mitotic cells significantly increased in 4OHT-treated transgenic PGC cultures (Table 1). As a result, the number of PGCs derived from the AKT-MER transgenic mice was significantly enhanced by adding 4OHT after 3 days of culture (Fig. 2C). Similarly, the formation of primary EG cell colonies, defined as the alkaline phosphatase-positive, multilayered colonies that appeared on day 5 of culture (Fig. 2B), was significantly enhanced by the presence of 4OHT in the transgenic PGC cultures (Fig. 2D). By contrast, no significant increase in EG cell colony formation was observed for the control PGCs.

To establish EG cell lines, whole wells of the primary cultures were passed onto SV/Sl⁴-m220 feeder cells and cultured for 5 days in the presence of LIF (Fig. 2A). Whole wells of the secondary cultures were then passed onto MEFs to establish EG cell lines. At day 5 of the tertiary culture, the wells containing EG cell colonies were counted to estimate the derivation efficiency. Both 4OHT and bFGF were omitted from the secondary and tertiary cultures, as the conversion to EG cells had been achieved in the primary culture (Matsui et al., 1992). As summarized in Table 2A, the efficiency of EG cell establishment from the transgenic PGCs was significantly enhanced when 4OHT was added to the primary culture. EG cell derivation was also enhanced when the migratory-phase PGCs at E8.5 were cultured with 4OHT (Table 2B). Conversely, the inhibition of PI3K signaling abrogated EG cell derivation from E11.5 PGCs (Table 3). However, AKT activation rescued EG cell derivation in the presence of PI3K inhibitor. These results indicate that PI3K/AKT signaling promotes the establishment of EG cell lines.

Replacement of bFGF and SCF signaling by AKT

We also examined whether AKT activation could substitute for bFGF, an essential growth factor for EG cell derivation (Matsui et al., 1992; Resnick et al., 1992). For this purpose, PGCs from E11.5 embryos were cultured in the presence of SCF and LIF, but in the absence of bFGF, during primary cultures. The percentage of apoptotic cells was significantly decreased in 4OHT-treated, transgenic PGC cultures (Table 1). Although mitotic PGCs were hardly detectable in the control cultures, mitotic cells were consistently observed in 4OHT-treated transgenic cultures (Table 1). At day 3 of primary culture, although the number of wild-type

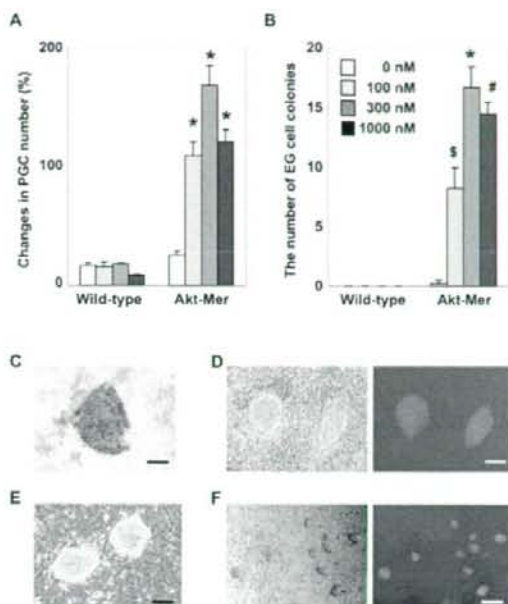


Fig. 3. EG cell production from AKT-MER-expressing PGCs in the absence of bFGF and SCF. (A) Increased number of AKT-MER-expressing PGCs in the absence of bFGF. E11.5 gonad cell suspensions were seeded onto SV/Sl⁴-m220 feeder cells and cultured with LIF but without bFGF. The cells of 0.1 embryos were added to each well. The percentage change in PGC number was calculated as described in Fig. 2C.

The 4OHT-treated transgenic PGCs proliferated significantly more than did the untreated transgenic PGCs (mean \pm s.e.m.; * P <0.005, Student's t -test; n =4) and wild-type PGCs cultured with the corresponding concentrations of 4OHT (P <0.005 for 100 nM, 300 nM and 1000 nM). (B) Primary EG cell colony formation by E11.5 PGCs of transgenic mice without bFGF. Significantly more EG colonies were generated in the 4OHT-treated transgenic cultures than in the untreated transgenic PGCs (mean \pm s.e.m.; * P <0.001, * P <0.005, * P <0.05, Student's t -test; n =4). (C) Alkaline phosphatase-positive primary EG cell colonies generated from transgenic PGCs treated with 300 nM 4OHT in the absence of bFGF. Cell suspensions of transgenic gonads at E11.5 were seeded onto SV/Sl⁴-m220 feeder cells and cultured with LIF alone for 5 days. Scale bar: 100 μ m. (D) Transgenic EG cell lines established without bFGF from the primary PGC cultures in C. The EG cells formed round, multilayered colonies (left) and were EGFP positive (right). Scale bar: 100 μ m. (E) EG cell lines established from E11.5 PGCs of transgenic mice without bFGF and SCF. Gonad cell suspensions were seeded onto MEFs, cultured with LIF and 300 nM 4OHT, and then passaged to tertiary cultures. Scale bar: 100 μ m. (F) Mixed culture of wild-type and transgenic cells. Gonad cell suspensions of E11.5 wild-type and transgenic mice were cultured on SV/Sl⁴-m220 feeder cells in the presence of LIF and 300 nM 4OHT, and passaged to tertiary cultures. All of the EG cell colonies were positive for EGFP (right). Scale bar: 250 μ m.

PGCs had decreased to less than 20%, the cultured PGCs of transgenic mice were restored by adding 4OHT (Fig. 3A). Similarly, although no EG cell colonies were detected for the wild-type PGCs, about 10% of the transgenic PGCs gave rise to primary EG cell colonies in the presence of 4OHT at day 5 of

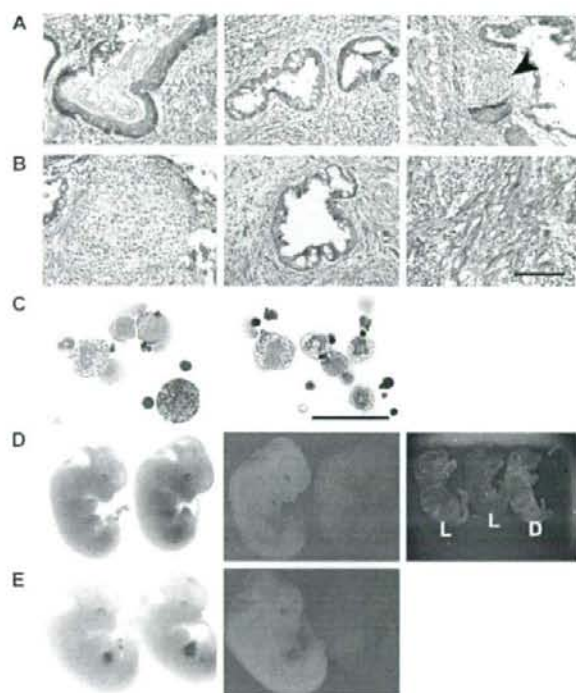


Fig. 4. Multipotent differentiation capacities of EG cells established in AKT-MER transgenic mice. (A) Hematoxylin and Eosin (HE) staining of teratomas derived from EG cells established without bFGF. The cells differentiated into various tissues, including squamous epithelium (left), glands (middle) and cartilage (right, arrowhead). (B) HE staining of teratomas derived from EG cells established without bFGF and SCF. The teratomas were composed of various tissues, including cartilage (left), mucosal glands (middle) and muscle (right). Scale bar: 100 μ m. (C) In vitro hematopoietic cell differentiation. EG cells established in the absence of bFGF (left), and the absence of bFGF and SCF (right), produced various hematopoietic cells in the OP9 differentiation system. Scale bar: 50 μ m. (D) Chimeras derived from the transgenic EG cells established without bFGF. The contribution of the EG cells could be assessed by monitoring the EGFP fluorescence. In the E12.5 embryos (left and middle), EGFP fluorescence was distributed in the entire body. Right, newborn non-chimeric (middle) and chimeric mice (left, right). L, live; D, dead at birth. Left chimera survived to adult. (E) Chimeras derived from transgenic EG cells established without bFGF and SCF. Shown is the E12.5 chimera.

culture (Fig. 3B,C). A few primary EG cell colonies emerged from the transgenic PGCs in the absence of 4OHT, presumably due to the leakiness of AKT-MER fusion protein activity in the absence of 4OHT (Fig. 1C).

To verify that AKT signaling in the primary culture did not merely trigger PGC proliferation but did induce EG cell formation, we examined whether the EG cell lines could be established via the above-mentioned three-step culture system (see Fig. 2A). When transgenic PGCs were cultured with 4OHT in the primary culture, EG cell lines were established in tertiary cultures with an efficiency that was comparable to that of wild-type PGCs cultured with bFGF (Fig. 3D, Table 2A). Although EG cell lines were also established from transgenic PGCs in the absence of 4OHT, the efficiency of this process was low. EG cell lines could also be established from 4OHT-treated E8.5 transgenic

PGCs in the absence of bFGF (Table 2B). Therefore, we conclude that AKT signaling activation can replace the effects of bFGF with respect to EG cell derivation.

It has been reported that treatment with bFGF during the first 24 hours of primary culture was sufficient for EG cell derivation (Durocova-Hills et al., 2006). To determine the effective time window of AKT signaling on EG cell derivation from transgenic mouse PGCs, AKT signaling was activated for 1 day only at various time points of primary culture, in the absence of bFGF (data not shown). When 4OHT was added for the first day and then removed for the remaining 4 days, EG cell lines were established efficiently from E11.5 transgenic PGCs. This efficiency was comparable to that achieved when 4OHT was added for the entire 5 days. As the level of AKT phosphorylation decreased to basal level within 24 hours of the removal of 4OHT

Table 4. Production of chimeric mice

EG cell line	Condition for EG cell establishment	No. of embryos transferred	Dead non-chimera	Dead chimera	Live non-chimera	Live chimera	Chimeras/total
M1-3	-bFGF, SCF, LIF	18	0	2	2	1*	3/5
		18	1	0	8	0	0/9
M1-5	-bFGF, SCF, LIF	22	0	1	2	2†	3/5
		26	1	2	2	0	2/5
M1-7	-bFGF, SCF, LIF	18	0	3	1	1‡	4/5
		28	0	0	0	0	0/0
L-2	-bFGF, -SCF, LIF	21	1	0	6	0	0/7
		18	0	0	2	0	0/2

*Chimeric mouse died soon after birth.

†One chimeric mouse survived to adulthood, whereas one chimera died soon after birth.

‡Chimeric mouse survived to adulthood.

Table 5. Effects of AKT activation on EG cell derivation from E13.5-E16.5 germ cells

Embryonic day	Sex	Forskolin	Seeded PGC (embryos/well)	4OHT (nM)	EG cell-positive wells		χ^2 test	
					Wild type	AKT-MER	Wild type	0 nM
E13.5	M	-	0.02	0	4/12	5/12	nsd	-
		-	0.02	300	5/12	12/12	<0.005	<0.005
	F	-	0.02	0	1/12	1/12	nsd	-
		-	0.02	300	2/12	2/12	nsd	nsd
	M	+	0.02	0	5/12	6/12	nsd	-
		+	0.02	300	6/12	12/12	<0.005	<0.005
	F	+	0.02	0	4/12	3/12	nsd	-
		+	0.02	300	3/12	8/12	<0.05	<0.05
E14.5	M	+	0.1	0	0/12	0/12	nsd	-
		+	0.1	100	0/12	5/12	<0.025	<0.025
		+	0.1	300	0/12	4/12	<0.05	<0.05
		+	0.1	1000	0/12	3/12	nsd	nsd
	F	+	0.1	0	0/5	0/5	nsd	-
		+	0.1	300	0/5	0/5	nsd	nsd
E15.5	M	+	0.1	0	0/10	0/10	nsd	-
		+	0.1	100	0/10	0/10	nsd	nsd
	F	+	0.1	0	0/5	0/10	nsd	-
		+	0.1	100	0/5	0/30	nsd	nsd
E16.5	M	+	0.1	0	0/12	0/12	nsd	-
		+	0.1	100	0/12	0/12	nsd	nsd
		+	0.1	0	0/12	0/12	nsd	-
	F	+	0.1	0	0/12	0/12	nsd	-
		+	0.1	100	0/12	0/12	nsd	nsd
		+	0.1	0	0/12	0/12	nsd	-

Cell suspensions from the gonads of E13.5 to E16.5 embryos were seeded onto *Sl/Si^h-m220* feeder cells and cultured with bFGF, LIF and forskolin in the presence of various concentrations of 4OHT. The cells of E13.5 embryos were also cultured without forskolin. M, male; F, female; nsd, not statistically different.

(data not shown), we conclude that AKT activation during the first 48 hours of primary culture was sufficient for EG cell derivation.

To examine whether AKT signaling could replace SCF signaling in the derivation of EG cells, we seeded E11.5 PGCs onto MEFs in the presence of LIF alone. As shown in Table 2A, although the efficiency was low, EG cell lines were established reproducibly from AKT-MER-expressing PGCs when 4OHT was added to the primary culture (Fig. 3E). By contrast, no EG cell colonies developed from wild-type PGCs or transgenic PGCs in the absence of 4OHT. The EG cell lines established without bFGF or SCF could be propagated indefinitely on MEFs in the presence of LIF.

Given that AKT-MER is expressed in germ cells and somatic cells, the enhanced EG cell derivation could be attributable to the supporting effects of gonadal somatic cells with high AKT activity. To exclude this possibility, mixtures of cells obtained from wild-type and transgenic gonads were cultured in the presence of SCF and LIF without bFGF. As shown in Fig. 3F, all of the established EG cells were transgenic PGC-derived, EGFP-positive cells. Therefore, enhanced EG cell establishment was the result of cell-autonomous effects of AKT signaling in the PGCs.

A unique property of pluripotent stem cells is the ability to differentiate into multiple cell lineages. Therefore, we investigated whether pluripotency was maintained in EG cells established from E11.5 PGCs by AKT activation instead of by bFGF and/or SCF signaling. When the EG cells were transplanted into nude mice, these cells produced teratomas that were composed of various differentiated cells (Fig. 4A,B). In an *in vitro* hematopoietic

differentiation system using OP9 stromal cells, these cells formed mesodermal colonies and subsequently produced a variety of hematopoietic cells on day 12 after the induction of differentiation (Fig. 4C). Furthermore, we injected the EG cells into the blastococcal to examine whether they had the ability to contribute to chimeric mice. When the EG cells established without bFGF were used, EGFP-positive EG cell-derived cells were detectable in the whole bodies of the E12.5 embryos, and live chimeric mice were born (Fig. 4D). The EG cells derived without bFGF or SCF also contributed to embryonic tissues, but live chimeras were not born (Fig. 4E). Some chimeras showed fetal overgrowth and were dead at birth (Table 4). The EG cells were not transmitted through the germ line. It is worthwhile to mention that germ-line transmission was reported in the literature for EG cells derived from E8.5 PGCs but not those derived from E11.5-E12.5 PGCs (Labosky et al., 1994; Stewart et al., 1994; Tada et al., 1998). Thus, AKT signaling produces EG cells with pluripotency from PGCs, in the absence of bFGF and SCF.

Derivation of EG cell lines from later embryonic stages

The ability to produce EG cells is lost progressively during germ cell differentiation. After E13.5, the germ cells enter mitotic arrest and meiosis in males and females, respectively (McLaren, 2003; Wylie, 2000). EG cell production from E13.5 transgenic germ cells cultured under standard conditions was enhanced by 4OHT in males but not in females (Table 5). Co-stimulation with bFGF and forskolin enhances EG cell formation (Koshimizu et al., 1996). In the presence of forskolin, 4OHT enhanced the efficiency of EG cell establishment from E13.5 PGCs of both males and females.

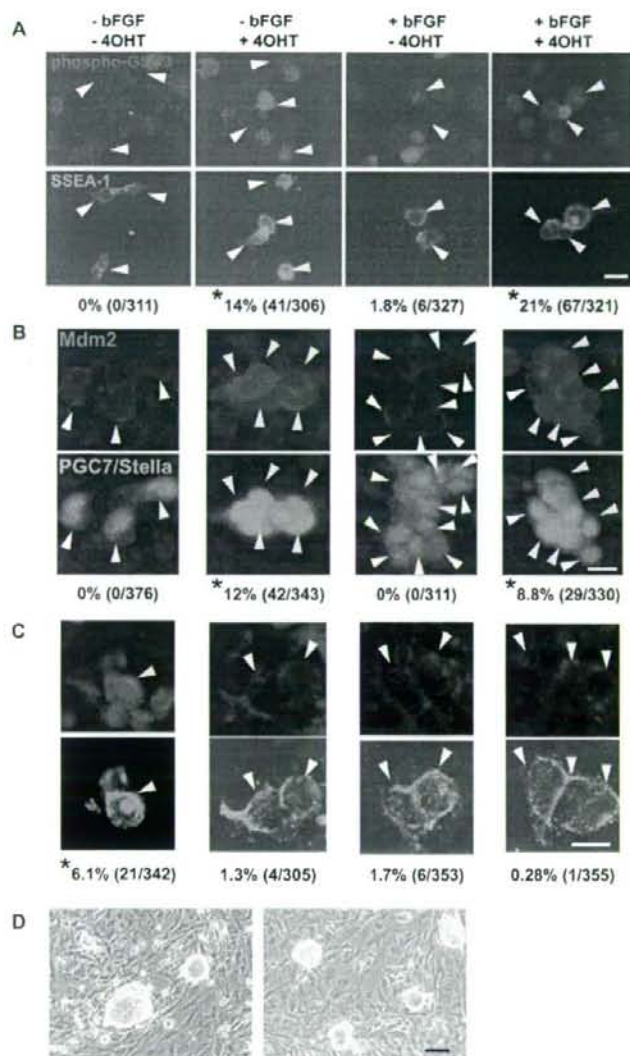


Fig. 5. The AKT downstream proteins and EG cell lines derived from p53-deficient mice.

(A–C) Immunostaining with antibodies against phospho-GSK3 (A), MDM2 (B) and Ser20-phospho-p53 (C). The AKT-MER transgenic PGCs were cultured for 2 days in the presence of the indicated reagents. The cells were stained with antibodies against AKT downstream molecules (red) and germ cell markers SSEA-1 or PGC7/Stella (green). Yellow and white arrowheads indicate the stained and unstained PGCs, respectively. The percentage of positive cells is shown below each picture. The percentage of phospho-GSK3-positive PGCs (A) and of PGCs stained strongly with MDM2 antibody (B) was significantly higher in the 4OHT-treated PGCs, irrespective of bFGF treatment ($*P < 0.005$, χ^2 test), than in wild type. The percentage of phospho-p53-positive PGCs cultured without 4OHT and bFGF was significantly higher than that of other groups ($*P < 0.005$, χ^2 test; C). More than 300 PGCs were analyzed in each group. Scale bars: 25 μ m. (D) EG cell lines derived from p53-deficient mice. EG cell lines were established in the presence (left) or absence (right) of bFGF. Scale bar: 100 μ m.

EG cells were not produced from E14.5 germ cells of either wild-type or transgenic mice in the absence of 4OHT. Only male E14.5 transgenic germ cells gave rise to EG cells in the presence of 4OHT, although with a much lower efficiency than from E13.5 germ cells. From E15.5, EG cell lines could not be established from either male or female germ cells. These results show that the efficiency of EG cell establishment declines earlier in females than in males, and that activation of AKT signaling cannot promote EG cell derivation from germ cells later than E14.5.

Enhanced EG cell derivation by P53 deficiency

AKT exerts its biological effects via the phosphorylation of various downstream targets (Brazil et al., 2004). We next examined the status of possible downstream proteins in the

cultured PGCs. The activity of glycogen synthase kinase 3 (GSK3) is inhibited by AKT via direct phosphorylation. Treatment with a GSK3 inhibitor, BIO (6-bromoindirubin-3'-oxime), supports ES cell pluripotency and improves the efficiency of EG cell derivation (Sato et al., 2004; Umehara et al., 2007). As shown in Fig. 5A, the percentage of transgenic PGCs stained with anti-phospho-GSK3 antibody was increased by AKT activation regardless of the bFGF treatment. However, the treatment of wild-type PGCs with BIO did not enhance EG cell derivation (Table 6A).

The tumor suppressor p53 is negatively regulated by AKT signaling in several ways. First, the stability and nuclear localization of MDM2, an E3 ubiquitin ligase for p53, are enhanced via phosphorylation by AKT (Feng et al., 2004; Mayo

Table 6. Effects of GSK3 inhibition and p53 deficiency on EG cell derivation from E11.5 PGCs

A				
Growth factors	Treatment	Seeded PGC (embryo/well)	EG cell-positive wells	χ^2 test DMSO
bFGF, SCF, LIF	DMSO	0.02	5/12	–
	meBIO	0.02	5/12	nsd
	BIO	0.02	5/12	nsd
SCF, LIF	DMSO	0.1	0/12	–
	meBIO	0.1	0/12	nsd
	BIO	0.1	0/12	nsd

B				
Genotype	Growth factors	Seeded PGC (embryo/well)	EG cell-positive wells	χ^2 test –/–
+/+ (n=2)	bFGF, SCF, LIF	0.02	1/12	<0.005
+/- (n=11)		0.02	9/66	<0.005
-/- (n=4)		0.02	22/24	
+/+ (n=5)	SCF, LIF	0.1	0/25	<0.01
+/- (n=12)		0.1	0/60	<0.005
-/- (n=7)		0.1	8/35	

(A) E11.5 wild-type PGCs were cultured with the GSK3 inhibitor BIO, its inactivated derivative Me-BIO (methyl-BIO) or DMSO during primary culture. EG cell derivation was carried out as described in Fig. 2A.

(B) p53 heterozygous females were crossed with heterozygous males. Gonads were isolated at E11.5. Cell suspensions from the individual embryos were seeded onto SV59-m220 feeder cells and cultured with growth factors as indicated.

and Donner, 2001; Zhou et al., 2001). As shown in Fig. 5B, only a faint MDM2 signal was detected in the cytoplasm of transgenic PGCs in the absence of 4OHT. However, the percentage of PGCs that showed stronger MDM2 staining, in both the cytoplasm and nucleus, increased in 4OHT-treated transgenic PGCs. Second, the Ser20 phosphorylation of p53 by checkpoint kinase CHK1/CHK2 (CHEK1/CHEK2 – Mouse Genome Informatics) is required to induce cell cycle arrest and apoptosis at the G2/M phase transition (Hirao et al., 2000; Shieh et al., 1999). It has been reported that PI3K/AKT signaling inhibits both the basal and the DNA damage-induced activity of CHK1/2 (Hirose et al., 2005; Shtivelman et al., 2002). In the absence of bFGF and 4OHT, about 6% of transgenic PGCs were stained with the anti-Ser20-phospho-p53 antibody (Fig. 5C). However, the percentage of phospho-p53-positive PGCs decreased after the addition of bFGF and/or 4OHT in the transgenic cultures (Fig. 5C). These results suggest that AKT signaling inhibited p53 activity in transgenic PGCs. To examine the effects of p53 inhibition on EG cell derivation, we used p53-deficient mice with a mixed background (C57BL/6, CBA2 and ICR). In the presence of bFGF, the efficiency of EG cell derivation from p53-deficient embryos was significantly higher than that from wild-type and heterozygous mice (Table 6B, Fig. 5D). In addition, EG cell lines were reproducibly established from p53-deficient mice even in the absence of bFGF.

DISCUSSION

We have demonstrated that AKT signaling, one of the downstream targets of PI3K, promotes EG cell derivation from PGCs. The deletion of *Pten* in PGCs induces early onset of testicular teratomas, in which AKT is hyperphosphorylated (Kimura et al., 2003). Furthermore, EG cell colony formation from E11.5 PGCs is enhanced in PGC-specific *Pten*-deficient mice, and by treatment with an antisense oligonucleotide against *Pten*, in the presence of three growth factors (Kimura et al., 2003; Moe-Behrens et al., 2003). The EG cell-forming efficiency of AKT-MER-expressing PGCs in the presence of 4OHT was comparable to that of PGC-specific *Pten*-deficient mice (Fig. 2C). Therefore, it is conceivable that AKT is the main downstream effector of PI3K that causes the phenotypes of *Pten*-deficient mice.

EG cell derivation from the PGCs of either *Pten*-deficient or wild-type mice requires bFGF (T.K. and T.N., unpublished), which suggests that the PI3K signaling activated by bFGF is crucial for EG cell derivation. Our finding that 4OHT-treated AKT-MER-expressing PGCs generate EG cell colonies without bFGF as efficiently as do wild-type PGCs cultured with bFGF (Table 2, Fig. 2D, Fig. 3B) demonstrates that AKT signaling activation can functionally replace bFGF. However, the EG cell-forming efficiency was lower than that of AKT-MER-expressing PGCs cultured in the presence of 4OHT and bFGF, or *Pten*-deficient PGCs cultured in the presence of bFGF. In addition, the effects of AKT activation in our system would be greater than those caused at a physiological level, because AKT phosphorylation in AKT-MER transgenic PGCs was higher than that in bFGF-treated wild-type PGCs (Fig. 1C). These results suggest that the PI3K/AKT signal cooperates with some other signaling pathways in EG cell derivation. Besides PI3K/AKT signaling, bFGF activates several intracellular signaling components, such as mitogen-activated protein kinase (MAPK) and phospholipase C γ (Klint and Claesson-Welsh, 1999). In addition, the proliferation of PGCs in culture is supported by MAPK signaling (De Miguel et al., 2002), which raises the possibility that the PI3K/AKT and MAPK signaling pathways promote EG cell derivation in a cooperative fashion.

The mitotic activity and survival of PGCs strongly correlate with the efficiency of EG cell formation and the incidence of testicular teratomas. Forskolin and retinoic acid, which can substitute for bFGF in EG cell derivation, are reported to act as mitogenic and survival factors for PGCs (Koshimizu et al., 1996; Koshimizu et al., 1995). Similarly, AKT activation also promoted proliferation and inhibited apoptosis in the cultured AKT-MER transgenic PGCs (Table 1). Despite the fact that male PGCs enter mitotic arrest at E13.5 in most strains of mice, the germ cells continue to proliferate until after E14.5 in mouse strains that are susceptible to testicular teratoma, such as 129/Sv-*Ter/Ter* and *Pten*-deficient mice (Kimura et al., 2003; Noguchi and Stevens, 1982). Although the majority of mitotic PGCs are eventually lost by apoptosis in these mouse strains, a population of PGCs survives to generate testicular teratomas. Thus, sustained proliferation and subsequent survival of PGCs may be a prerequisite for EG cell establishment.

The potential to develop EG cells from germ cells is completely lost at E14.5, which is coincident with the emergence of mitotic arrest and meiosis of male and female germ cells, respectively. Responsiveness to AKT signaling for EG cell establishment was not detectable after E14.5 and E15.5 in females and males, respectively (Table 5). In contrast to the E11.5 PGCs, the E15.5 transgenic germ cells could not resume proliferation following AKT activation (data not shown). The effects of AKT signaling diminished earlier in females than in males, which could be due to the small number of mitotic germ cells that remain in E14.5 male gonads (Kimura et al., 2003). Thus, mitotic responsiveness to AKT signaling may be crucial for enhancing EG cell establishment. At the same time, AKT signaling promoted self-renewing cell division of spermatogonial stem cells but not derivation of pluripotent cells from spermatogonial stem cells (Lee et al., 2007). These results show that AKT provoked distinct responses in germ cells depending on the developmental stage.

PI3K/AKT signaling regulates a variety of downstream molecules, some of which play crucial roles in various stem cell systems. In this study, we have shown that AKT signaling negatively regulated p53 in the cultured PGCs by enhanced MDM2 function, and inhibited phosphorylation of p53. We also revealed that EG cell derivation was promoted by the absence of p53. Thus, AKT-mediated p53 inhibition would be important to promote the EG cell derivation. Similarly, derivation of pluripotent cells from spermatogonial stem cells is enhanced by the p53 deficiency (Kanatsu-Shinohara et al., 2004). Therefore, suppression of p53 may be crucial for germ cells to acquire pluripotency. Consistently, the activity and nuclear translocation of p53 is suppressed in the pluripotent ES cells (Aladjem et al., 1998), and differentiation of ES cells is induced by p53 through suppression of *Nanog* expression (Lin et al., 2005). The p53 deficiency in the cultured germ cells may promote the acquisition of undifferentiated states by altering the expression of the target genes of p53. Meanwhile, considering that AKT signaling regulates other downstream molecules, such as mTOR complex 1, it is likely that AKT signaling promotes EG cell derivation through multiple downstream molecules. Further analysis would not only provide an efficient means for establishing germ cell-derived stem cells, but also give valuable insights into the developmental plasticity of the germ cells.

We thank Drs N. Tanaka and E. Morii for providing p53-deficient mice and histological inspection. We also thank Dr M. Okabe, Ms A. Kawai and Ms Y. Koreeda for the production of chimeric mice. SCF was kindly provided by Amgen. This work was supported in part by grants from the Ministry of Education, Science, Sports, and Culture, Uehara Memorial Foundation, Astellas Foundation for Research on Metabolic Disorders, and the 21st Century COE 'CICET'.

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blood

2008 111: 1924-1932
Prepublished online Dec 6, 2007;
doi:10.1182/blood-2007-08-104489

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Differential context-dependent effects of friend of GATA-1 (FOG-1) on mast-cell development and differentiation

Daijiro Sugiyama,¹ Makoto Tanaka,¹ Kenji Kitajima,¹ Jie Zheng,¹ Hilo Yen,¹ Tomotaka Murotani,² Atsushi Yamatodani,² and Toru Nakano¹

¹Department of Stem Cell Pathology, Medical School, and Graduate School of Frontier Biosciences and ²Department of Medical Physics and Engineering, Division of Health Sciences, Graduate School of Medicine, Osaka University, Japan

Friend of GATA-1 (FOG-1) is a binding partner of GATA-1, a zinc finger transcription factor with crucial roles in erythroid, megakaryocytic, and mast-cell differentiation. FOG-1 is indispensable for the function of GATA-1 during erythro/megakaryopoiesis, but FOG-1 is not expressed in mast cells. Here, we analyzed the role of FOG-1 in mast-cell differentiation using a combined experimental system with conditional gene expression and *in vitro* hematopoietic induction of mouse embryonic stem cells. Expression of FOG-1

during the progenitor period inhibited the differentiation of mast cells and enhanced the differentiation of neutrophils. Analysis using a mutant of PU.1, a transcription factor that positively or negatively cooperates with GATA-1, revealed that this lineage skewing was caused by disrupted binding between GATA-1 and PU.1, which is a prerequisite for mast-cell differentiation. However, FOG-1 expression in mature mast cells brought approximately a reversible loss of the mast-cell phenotype. In contrast to the lineage skewing,

the loss of the mast-cell phenotype was caused by down-regulation of *MITF*, a basic helix-loop-helix transcription factor required for mast-cell differentiation and maturation. These results indicate that FOG-1 inhibits mast-cell differentiation in a differentiation stage-dependent manner, and its effects are produced via different molecular mechanisms. (Blood. 2008; 111:1924-1932)

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Introduction

In hematopoiesis, more than 10 lineages of mature blood cells are derived from a single hematopoietic stem cell.¹ This process is tightly regulated by extrinsic environmental cues and an intrinsic genetic program. Multipotent hematopoietic cells gradually lose their differentiation ability, giving rise to lineage-restricted hematopoietic progenitors. Once matured, each type of blood cell expresses a set of lineage-specific genes that supports its physiologic functions. Several lineage-specific transcription factors have been identified that control lineage-specific gene expression, and recent studies have demonstrated that these transcription factors cooperate with other transcription factors and cofactors. Moreover, these lineage-specific transcription factors display a variety of biologic functions in a cellular context-dependent manner.

The GATA family is the most extensively studied group of hematopoietic transcription factors. All GATA factors recognize a specific DNA sequence known as a GATA box [(A/T)GATA(A/G)]² using the C-finger domain in one of their 2 zinc fingers (N- and C-fingers). *GATA-1*, a founding member of the GATA family, is highly expressed in erythroid cells, megakaryocytes, mast cells, and eosinophils.^{3,4} Gene targeting analyses have shown that *GATA-1* is essential for terminal differentiation in erythropoiesis and megakaryopoiesis.⁵⁻⁸ Deletion of the upstream regulatory elements in *GATA-1* gene leads to reduced *GATA-1* expression (*GATA-1^{low}*),⁹ indicating that the gene plays a pivotal role in mast-cell differentiation. In *GATA-1^{low}* mice, morphologically abnormal mast cells were observed in the connective tissue and

peritoneal lavage. Another GATA family member, *GATA-2*, also participates in mast-cell development.¹⁰

Yeast 2-hybrid screening revealed that friend of GATA-1 (FOG-1) binds the N-finger of GATA-1.¹¹ FOG-1 is highly expressed in erythroid and megakaryocytic cells but not in mast cells.¹¹ Gene targeting analysis revealed that FOG-1 is required for erythroid and megakaryocyte development and that it is essential for the physiologic function of *GATA-1* in those lineages.¹²⁻¹⁴ FOG-1 has complex effects on gene expression, because it functions as both a coactivator and a corepressor of GATA-1 and GATA-2. For example, activation of the megakaryocyte-specific *αIIb* gene by GATA-1 is enhanced by FOG-1; however, the GATA-1/FOG-1 complex represses *GATA-2* expression in erythroid cells.¹⁵⁻¹⁷

The lack of FOG-1 expression in mast cells suggests that it may inhibit mast cell-specific gene expression and mast-cell differentiation. To examine the effects of FOG-1 expression during mast-cell differentiation, we used *in vitro* hematopoietic differentiation of mouse embryonic stem (ES) cells. During coculture of ES cells with OP9 stroma cells (OP9 system), the interleukin-3 (IL-3) preferentially induces mast-cell differentiation. The OP9 system, in combination with a conditional gene expression system based on tetracycline (TET; OP9-TET system), is a powerful tool for analyzing gene function during hematopoiesis.¹⁸⁻²² We previously established ES cell lines conditionally expressing exogenous FOG-1 and analyzed FOG-1 function during erythroid and

Submitted August 2, 2007; accepted November 19, 2007. Prepublished online as Blood First Edition Paper, December 6, 2007; DOI 10.1182/blood-2007-08-104489.

The online version of this article contains a data supplement.

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megakaryocytic differentiation.²⁰ *FOG-1* suppressed the proliferation of erythroid and early megakaryocytic cells but it enhanced the proliferation of megakaryocytic cells at later stages of development. In other words, *FOG-1* functioned in a cell context-dependent manner during erythroid and megakaryocytic differentiation.

In this study, we found that *FOG-1* expression inhibited mast-cell differentiation in a stage-dependent manner via different molecular mechanisms. *FOG-1* inhibited the differentiation of mast cells when expressed during the progenitor stage, instead favoring the development of neutrophils. In contrast, *FOG-1* expression in mature mast cells resulted in reduced numbers of cytoplasmic granules and a loss of mast cell-specific gene expression. Thus, our findings show that *FOG-1* inhibits mast cell differentiation in a cell context-dependent manner.

Methods

Cell culture

E14g2a ES cells and their derivatives were used in this study. The TET-regulated FOG-1 ES cells (TET-FOG-1 ES cells) used in this study have been described previously.²⁰ OP9 stroma cells, the retrovirus-producing packaging cell line Plat-E (a kind gift from Dr T. Kitamura, Tokyo University, Tokyo, Japan) and 293T cells were maintained as described previously.^{21,22} ES cells were transferred onto OP9 stroma cells in 6-well plates at a density of 10^4 cells/well. The induced cells were trypsinized on day 5, and 10^5 cells were seeded onto fresh OP9 cells. IL-3 produced by the X63 melanoma cell line, which expresses murine IL-3 (a gift from Dr H. Karasuyama, Tokyo Metropolitan University, Tokyo, Japan) was added from day 5 to induce mast-cell differentiation. On day 14, the mature mast cells were transferred to a suspension culture and maintained in IL-3 and stem cell factor (SCF; a kind gift from Amgen, Thousand Oaks, CA).

Flow cytometer

The primary antibodies used were biotin-GR-1, biotin-MAC-1 (BD Biosciences, Franklin Lakes, NJ), PE/Cy7-c-KIT (BD Biosciences), and PE-CD71 (eBioscience, San Diego, CA). The biotinylated antibodies were visualized with phycoerythrin/Cy5-conjugated streptavidin (BD Biosciences) using a procedure described previously.²¹ Cell sorting and analysis were performed using FACSCalibur and FACSAria flow cytometer (BD Biosciences).

Morphologic analysis

The cells were cytocentrifuged (approximately 5×10^4 cells/slide) at 600 rpm (45g) for 4 minutes with a Cytospin 4 (Thermo Fisher Scientific, Runcorn, Cheshire, United Kingdom) and then stained with May-Grünwald solution followed by Giemsa solution (Nacal Tesque, Kyoto, Japan). Images were obtained using an Olympus Provis microscope with a UPlan APO 40 \times /0.85 numeric aperture objective lens, an Olympus DP70 camera, and DC controller software (all from Olympus, Tokyo, Japan) at room temperature in air. The images were processed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Colony-forming assay

The hematopoietic cells present after 8 days of differentiation in the presence of IL-3 were recovered by thorough pipetting. The floating cells were collected and transferred to methylcellulose culture medium (Methocult M3232; Stem Cell Technology, Vancouver, BC, Canada) containing IL-3. After 7 days, individual colonies were selected, cytocentrifuged and inspected for the presence of each lineage of hematopoietic cells.

Plasmids

Mutant *PU.1* (*PU.1ΔC*) was constructed by amplifying the *PU.1* N-terminal region (from the translation initiation site [+1] to +513) from

pBS-PU.1. pBS-PU.1, which contains full-length murine *PU.1* cDNA, was a kind gift from Dr T. Oikawa (Sasaki Institute, Tokyo, Japan). The primers used were (5'-GTC GAC GGG TTA CAG GCG TGC AAA ATG-3') and antisense (5'-TTA CTT TTT CTT GCT GCC TGT CTC-3'). The amplified *PU.1* fragment was then sub-cloned into pGEM-T (Promega, Madison, WI), and its sequence was confirmed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA). The *PU.1ΔC* fragment, excised by SalI-NotI, was subsequently introduced into the SalI-NotI sites of pME2-FLAG, a gift from Dr K. Ohishi (Osaka University, Osaka, Japan). The *FLAG-PU.1ΔC* fragment excised by EcoRI-NotI was introduced into the EcoRI-NotI sites of the retroviral vector *pMY-IRES-EGFP*, a gift from Dr T. Kitamura. The XhoI-BsrGI fragment, containing *FLAG-PU.1ΔC* excised from *pMY-FLAG-PU.1ΔC-IRES-EGFP*, was introduced into the XhoI-BsrGI sites of the lentiviral vector *pLV-CAG-IRES-EGFP*, a gift from Dr M. Ikawa (Osaka University, Osaka, Japan). The cDNAs encoding a dominant-negative version of microphthalmia-associated transcription factor (*miMITF*), which lacks one of 4 consecutive arginines in the basic domain and wild-type *MITF* were provided by Dr E. Morii (Osaka University, Osaka, Japan). *pLV-IRES-EYFP* was constructed by replacing *EGFP* with *EYFP* (from *pEYFP*, BD Biosciences). The *miMITF* and *MITF* cDNAs were inserted into *pLV-IRES-EYFP* to create *pLV-miMITF-IRES-EYFP* and *pLV-MITF-IRES-EYFP*, respectively.

Retrovirus and lentivirus infection

pMY-IRES-EGFP and *pMY-FLAG-PU.1ΔC-IRES-EGFP* were transfected into Plat-E cells using Lipofectamine 2000 (Invitrogen, Rockville, MD), as described previously.²¹ Lentivirus particles were produced by transient cotransfection of the lentiviral plasmids with *pCMV-ΔR8.91* and *pMD2G-VSV-G* into 293T cells using Lipofectamine 2000. The retroviral and lentiviral infections were performed by centrifugal inoculation as described previously.²¹

RT-PCR analysis

Total RNA was recovered using the RNeasy Mini Kit (QIAGEN, Valencia, CA). cDNA synthesis was performed using the ThermoScript reverse transcription-polymerase chain reaction (RT-PCR) system (Invitrogen, Carlsbad, CA) with a random hexamer, as recommended by the manufacturer. The primers and conditions used to amplify *GATA-1*, *FOG-1*, *PU.1*, *c-KIT*, *Fcε receptor β chain (FceRβ)*, mouse mast cell protease, *MMCP-1* (*p1*), *-4* (*p4*), *-5* (*p5*), *-6* (*p6*), *granzyme B* (*granB*), *carboxypeptidase A* (*cpa*), *tryptophan hydroxylase* (*tph*), *MITF*, and *glyceraldehyde dehydrogenase* (*gapdh*) will be provided upon request.

Immunoprecipitation and Western blotting

Various combinations of the *GATA-1*, *PU.1*, and *FOG-1* expression vectors were transfected into 293T cells using a calcium phosphate. The expression vectors for *GATA-1* (*pEF-GATA-1*), *PU.1* (*pEF-PU.1*), and *FOG-1* (*pMT2-FOG-1*) were gifts from Dr M. Yamamoto (Tohoku University, Sendai, Japan), and Drs T. Oikawa and S.H. Orkin (Children's Hospital, Boston, MA). The cells were harvested 48 hours after transfection, and nuclear extracts were prepared. In brief, the cells were suspended in buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.1 mM ZnCl₂, and Complete protease inhibitor cocktail [Roche, Basel, Switzerland]) and centrifuged at 3000g for 10 minutes at 4°C. The pellets were then resuspended in buffer B (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 2 mM EDTA, 0.5 mM DTT, 0.1 mM ZnCl₂, and Complete protease inhibitor cocktail) and incubated on ice for 20 minutes. After centrifugation at 5000g for 10 minutes at 4°C, the supernatants were collected. The nuclear extracts were precleared using Protein-G Sepharose (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom) for 120 minutes at 4°C, followed by incubation with rat anti-GATA-1 antibodies (N6; Santa Cruz, CA) overnight at 4°C. Immunoprecipitations were performed in immunoprecipitation (IP) buffer [150 mM NaCl, 0.1% Nonidet P40, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 mM ZnCl₂, Complete] for 4 hours at 4°C using Protein-G Sepharose beads. The immunoprecipitants were then

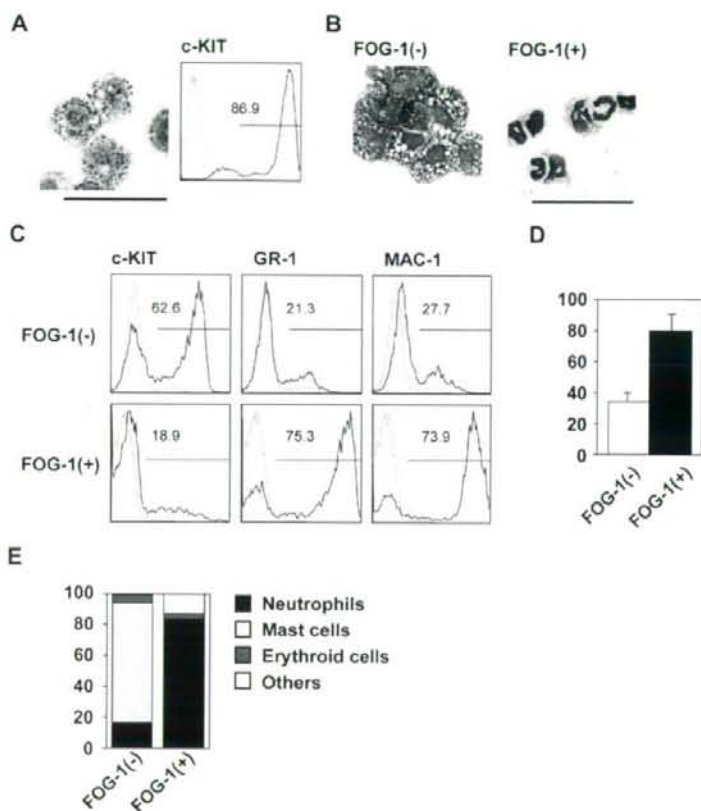


Figure 1. Lineage switch after *FOG-1* expression in progenitor cells. (A) Giemsa staining and FACS analysis on day 14 of E14tg2a ES cells in the presence of IL-3 since day 5. Antibody staining (blue line) and IgG control staining (gray line) were shown. (B) Giemsa staining of day 14 cells derived from TET-*FOG-1* ES cells. Differentiation was induced in TET-*FOG-1* ES cells without *FOG-1* expression until day 8. IL-3 was added from day 5, and *FOG-1* was induced on day 8. (C) FACS analysis of day 14-induced cells stained with anti-c-KIT, GR-1, and MAC-1 antibodies. The culture conditions were the same as in (B). (D) The total number of day 14 cells differentiated from TET-*FOG-1* ES cells. Error bars represent SEM. (E) Percentage of each type of colony. The day 8 cells induced from TET-*FOG-1* ES cells without *FOG-1* expression were transferred to methylcellulose media containing IL-3, and *FOG-1* expression was induced. Individual colonies were selected and analyzed after 7 days of culture. Bars represent 50 μ m.

washed with IP buffer, and the bound materials were eluted by boiling in 1 \times Laemmli buffer. The proteins were resolved by 4% to 20% SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Billerica, MA). The membranes were probed with antibodies to GATA-1 (N6), *FOG-1* (M-20), and PU.1 (T-21), (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA), or FLAG (M1; Sigma, St Louis, MO) followed by staining with HRP-conjugated anti-rat or anti-rabbit antibodies (Zymed, South San Francisco, CA). Antibody staining was visualized using the enhanced chemiluminescence (ECL) Detection Kit (GE Healthcare).

Reporter assay

The reporter TK-100-PU \times 3-Luc carrying 3 synthetic ETS recognition sites (a gift from Dr T. Oikawa),²⁴ and pRL-TK, carrying the *Renilla reniformis* luciferase gene, were cotransfected with various combinations of the *PU.1*, *GATA-1*, *FOG-1*, and/or *PU.1* Δ C expression vectors into CV-1 cells using a calcium phosphate.¹⁹ Cell lysates were prepared 48 hours after transfection, and reporter activity was measured using the Dual Luciferase Reporter Kit (Promega, Madison, WI). The transfection efficiencies were normalized by RL-TK activity.

Results

The effects of *FOG-1* on mast-cell lineage determination

We first examined the biologic effects of *FOG-1* on early mast-cell differentiation using an in vitro differentiation system in which

mouse ES cells were cultured on OP9 cells in the presence of IL-3. On day 8, the hematopoietic cells had become immature myeloid progenitors; by day 14, more than 80% of the differentiated cells exhibited the basophilic granules characteristic of c-KIT^{high} mast cells (Figure 1A). These cells efficiently released histamine upon crosslinking IgE receptors (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), showing that the cells were functionally differentiated mast cells. In contrast, those cells in which *FOG-1* was expressed beginning on day 8 by the withdrawal of tetracycline were quite different from the controls at day 14. More than 75% of the cells had the morphology of neutrophils, which express GR-1 and MAC-1 (Figure 1B,C). In addition, phagocytosis assay showed that the *FOG-1* induced GR-1⁺ cells possessed phagocytotic activity (Figure S2). These data indicate that *FOG-1* expression induced the neutrophils characterized by morphology, surface marker expression, and phagocytotic function. Because the total number of cells was increased more than 2 fold by *FOG-1* (Figure 1D), the increased percentage was due to a rise in the number of neutrophils and a decrease in the number of mast cells.

The observed increase may be explained by enhanced proliferation of the neutrophil progenitors or by the preferential differentiation of neutrophils. We used methylcellulose colony assays to discriminate between these 2 possibilities. Cells were differentiated on day 8 with tetracycline and transferred to methylcellulose media containing IL-3 in the presence or absence of tetracycline. As

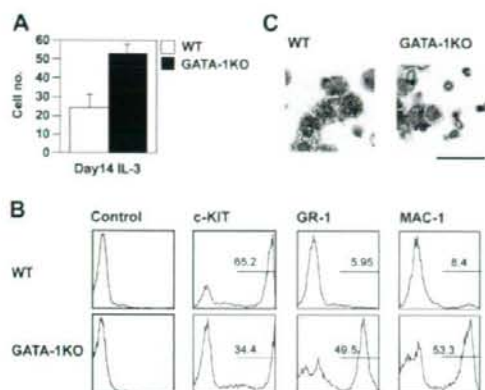


Figure 2. Preferential differentiation of GATA-1-null ES cells into neutrophils. (A) Total number of day 14-induced cells from E14tg2a and GATA-1-null ES cells exposed to IL-3 since day 8. Error bars represent SEM. (B) FACS analysis of day 14-induced cells. The cells were stained with anti-c-KIT, GR-1, and MAC-1 antibodies. Antibody staining and IgG control staining were shown. (C) Giemsa staining of the day 14-induced cells. Bar represents 50 μ m.

shown in Figure 1E, *FOG-1* drastically increased the percentage of neutrophil colonies. Because the total colony numbers were similar between the 2 groups (data not shown), the data indicate that *FOG-1* induced the development of the mast cells into neutrophils at the progenitor level. Therefore, we conclude that *FOG-1*-induced differentiation of mast cells into neutrophils occurs when

the gene is expressed during the progenitor phase. In particular, we found that expressing *FOG-1* on day 10 induced neutrophilic differentiation (data not shown).

Lineage skewing by abrogation of the interaction between GATA and PU.1

Because *FOG-1* is a necessary cofactor of *GATA-1* during erythropoiesis, we compared the proportions of mast cells and neutrophils produced from wild-type ES cells and *GATA-1*-null ES cells. Based on our cell count and fluorescence-activated cell sorting (FACS) data (Figure 2A,B), the *GATA-1*-deficient ES cells were able to differentiate into mast cells; however, the loss of *GATA-1* caused an increase in the number of neutrophils (Figure 2C), similar to the results of *FOG-1* overexpression. This suggests that *FOG-1* overexpression inhibited function of *GATA-1* critical for the differentiation of mast cells or neutrophils.

GATA-1 and *GATA-2* function cooperatively with PU.1 during mast-cell development.^{25,26} We hypothesized that *FOG-1* alters the physical interaction between the GATA factors and PU.1, thereby affecting differentiation. Thus, *GATA-1*, PU.1, and *FOG-1* were expressed together in 293T cells, and the effect of *FOG-1* on *GATA-1* and PU.1 complex formation was analyzed. As shown in Figure 3A, complex formation was disrupted by *FOG-1*. We next analyzed whether *FOG-1* relieves the inhibitory effect of *GATA-1* on PU.1. As shown in Figure 3B, *GATA-1*-induced inactivation of PU.1 was reversed by *FOG-1*.

Based on these data, we tested whether disrupting the association between PU.1 and the GATA factors by another method would

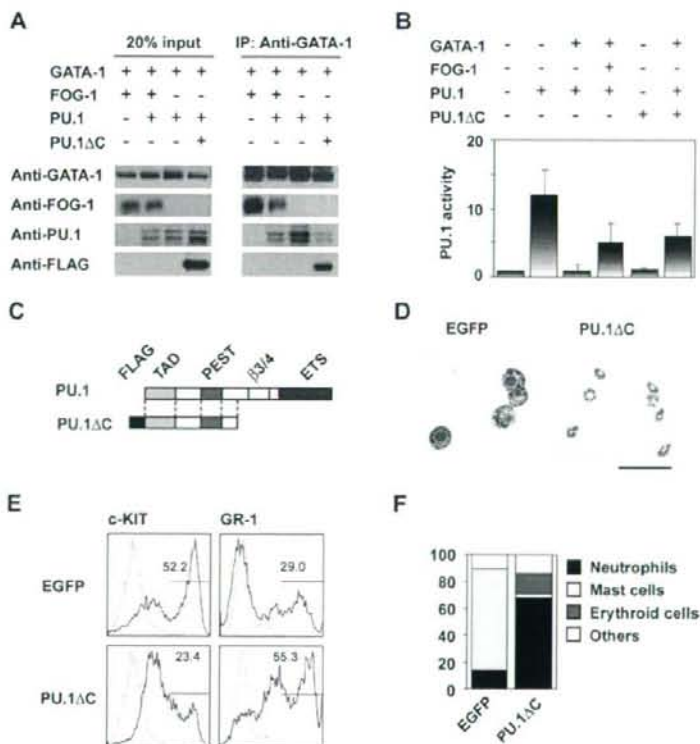


Figure 3. Inhibition of the interaction between GATA-1 and PU.1 by FOG-1 and PU.1ΔC. (A) Immunoprecipitation assays. 293T cells were cotransfected with various combinations of *GATA-1*, *FOG-1*, *PU.1*, and *PU.1ΔC* as shown. Immunoprecipitation was carried out using anti-GATA-1 antibodies. (B) Reporter assays using *TK-100-PU.1* × 3-Luc, which contains 3 synthetic ETS recognition sites. CV-1 cells were cotransfected with various combinations of *GATA-1*, *FOG-1*, *PU.1*, and *PU.1ΔC* as shown and with the PU.1-responsive reporter construct and the *RL-TK* control reporter. The relative activities, normalized by *RL-TK* activity, are shown. Error bars represent SEM. (C) Structure of PU.1 and PU.1ΔC. (D) May-Giemsa staining of day 14-induced cells infected with *MY-IRES-EGFP* (EGFP) and *MY-PU.1ΔC-IRES-EGFP* (PU.1ΔC) on day 8. The EGFP⁺ cells were sorted. Bar represents 50 μ m. (E) FACS analysis of day 14-induced EGFP⁺ cells stained with anti-c-KIT and GR-1 antibodies. Antibody staining (blue line) and IgG control staining (gray line) were shown. (F) Percentage of each type of colony. Day-8 induced cells were infected with *MY-IRES-EGFP* and *MY-PU.1ΔC-IRES-EGFP*, and the EGFP⁺ cells were sorted on day 10. The sorted cells were transferred to methylcellulose media containing IL-3. Single colonies were selected and analyzed after 7 days of culture.

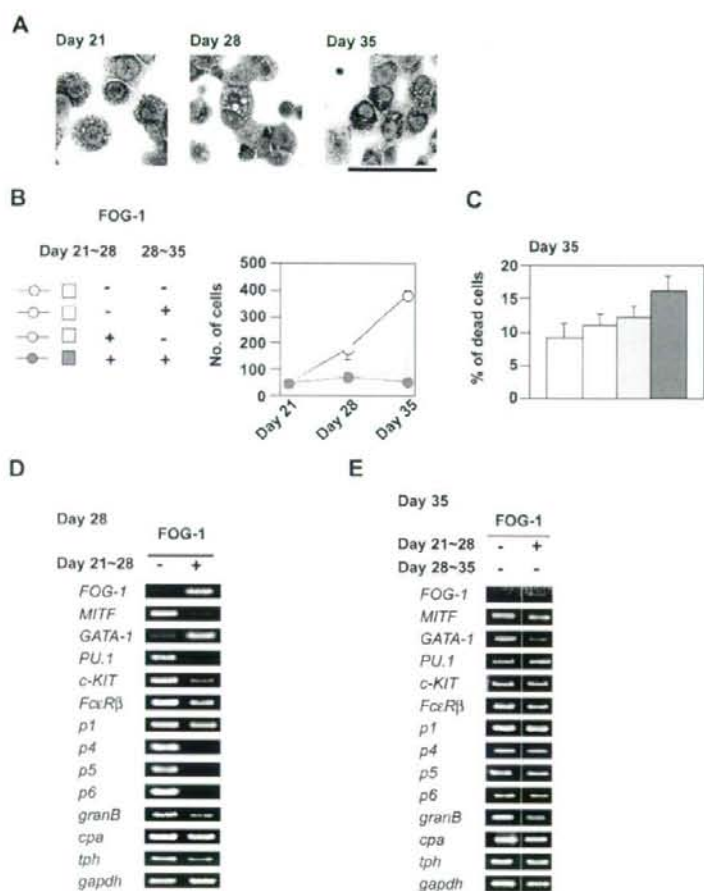


Figure 4. Inhibitory effects of *FOG-1* expression on mature mast cells. (A) Giemsa staining of mast cells. Day 21 indicates mast cells differentiated without expression of *FOG-1*. Day 28 and Day 35 indicate mast cells in which *FOG-1* was expressed between days 21 and 28, respectively. Bar represents 50 μ m. (B) Total numbers of cells. The duration of *FOG-1* expression is shown in the left panel. (C) The percentage of dead cells at day 35. The duration of *FOG-1* expression is as shown in panel B. Error bars represent SEM. (D,E) RT-PCR analysis of transcription factors and mast cell-specific genes.

result in a phenotype similar to that caused by *FOG-1* overexpression. *PU.1* contains an N-terminal transcriptional activation domain, through which it interacts with GATA proteins, a PEST domain, a β 3/4 domain, and a C-terminal DNA-binding ETS domain.^{27,28} We thus designed *PU.1 Δ C*, a C-terminal-truncated version of *PU.1* (Figure 3C). We expected that *PU.1 Δ C* would be unable to induce transcription since it lacked the ETS DNA binding domain, but that it would be able to bind to GATA factors. As expected, *PU.1 Δ C* inhibited the interaction of GATA-1 with *PU.1* (Figure 3A), and it relieved GATA-1-induced *PU.1* inactivation similar to *FOG-1* (Figure 3B). Meanwhile, *PU.1 Δ C* alone did not induce expression of the reporter.

To analyze the biologic effects of *PU.1 Δ C*, cells induced on day 8 were infected with *PU.1 Δ C-IRES-EGFP* retroviral vector and *IRES-EGFP*. The EGFP⁺ cells were sorted, cultured in the presence of IL-3, and analyzed on day 14. Compared with the control cells, greater numbers of neutrophils were observed among those cells expressing *PU.1 Δ C* (Figure 3D). As was the case for *FOG-1* overexpression, the GR-1⁺ population increased and the c-KIT^{high} population significantly decreased (Figure 3E). Colony formation assays of the EGFP⁺ cells on day 10 confirmed that *PU.1 Δ C* induced the switch from mast cells to neutrophils at the progenitor level (Figure 3F). These data

indicate that *PU.1 Δ C* has effects similar to those of *FOG-1* when introduced at the progenitor stage.

The effects of *FOG-1* on the phenotype of mature mast cells

We next examined the effects of *FOG-1* on fully differentiated mast cells, derived from TET-*FOG-1* ES cells, in the presence of IL-3 and tetracycline. The cells were further expanded in a liquid culture containing IL-3, SCF, and tetracycline until day 21 (Figure 4A left panel). *FOG-1* expression, which was induced between days 21 and 28, brought about a loss of basophilic granules (Figure 4A, middle panel). When *FOG-1* expression was ceased at day 28 and the cells were further cultured for 7 days, the morphologically normal mast cells reappeared (Figure 4A right panel). It is unlikely that the reappearance of normal mast cells was due to expansion from a minor population of granule-containing mast cells remaining on day 21, because cell growth was not significantly reduced by *FOG-1* expression (Figure 4B), and no significant cell death was induced (Figure 4C). It is plausible, therefore, that the loss of basophilic granules in the mast cells was reversible.

We used RT-PCR analysis to quantify the levels of transcription factor and mast cell-specific gene expression. *FOG-1* reduced the mRNA expression of *MITF*, *PU.1*, *c-KIT*, *Fc ϵ receptor β chain*