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Original Article

Methyl-CpG-binding domain 3 (Mbd3) is an important regulator for apoptosis in mouse embryonic stem cells

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Abstract: Methyl-CpG-binding domain 3 (Mbd3) is a core repressor complex component. Although Mbd3 is required for the pluripotency of embryonic stem cells (ES), the role of Mbd3 in mouse ES (mES) cell apoptosis remains undefined. In this study naïve-state mES were derived and maintained in the presence of a selective protein kinase C pathway inhibitor (PKCi; Gö6983) to study the function of Mbd3 during mES apoptosis. Mbd3 overexpression in mES decreased the total cell number and viability, and it also dramatically increased the rate of apoptosis. Further investigation of Mbd3 overexpression revealed a 3-fold increase in the proapoptotic/prosurvival protein ratio (Bax/Bcl-2) and elevated RNA expression levels of apoptosis-related genes, including *Bim*, *Trail*, *Fasl,* and *caspase 3,* with reduced *Bcl-2* RNA expression levels. Removal of PKCi from the mES cell culture resulted in upregulated Mbd3 expression and apoptosis, similar to the effects of Mbd3 overexpression. Furthermore, specific knockdown of endogenous Mbd3 partially rescued the mES apoptosis induced by the removal of PKCi, thus increasing the total cell number and viability while decreasing the rate of apoptosis. Additionally, *Bax*, *Bim*, *Trail,* and *caspase 3* RNA expression levels were partially reduced, and that of *Bcl-2* was partially increased. Our findings support Mbd3 as a pivotal regulator of apoptosis in mES.

Keywords: Mouse embryonic stem cells, protein kinase C, Mbd3, cell apoptosis, PKC inhibitor

Introduction

Embryonic stem cells (ES) are pluripotent cells derived from the inner cell mass of preimplantation blastocysts, and mouse ES (mES) were first derived by Evans and Kaufman in 1981 [1]. ES cell pluripotency is defined as a naïve and primed state [2]. Although many different stem cell lines have been derived over the years, only mES and rat ES (rES) have the capability of germline transmission and are thus referred to as naïve ES. It has been reported that mES can be derived and maintained with leukemia inhibitory factor (LIF), together with the inhibition of the mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3 (GSK3) pathways via selective small molecule inhibitors (termed 2iL), which elicit full pluripotency, including the capacity for germline transmission [3, 4]. The protein kinase C (PKC) family has been considered an intracellular mediator of several hormones, neurotransmitters, and tumor promoters, and it plays a vital role in growth regulation, differentiation, neurotransmission, and cell death [5]. It has been reported that inhibition of PKCζ/nuclear factor kappa beta (NF-κB) signaling with PKCi to block the microRNA-21/microRNA-29 axis supports mES and rES cell self-renewal [6, 7].

Apoptosis or "programmed cell death" is essential for embryonic development and plays an important role in the maintenance of cellular homeostasis. There are two [8] main apoptotic pathways: the intrinsic pathway mediated by mitochondria and the extrinsic pathway that relies on death-receptor signaling. Both pathways lead to the activation of caspase 3, which mediates cell death through additional cell damage [9, 10]. In mammalian cells, death-receptor signaling activates caspase 8, which further activates downstream effector caspase 3, leading to cellular apoptotic commitment [11]. However, in the mitochondria-mediated pathway, the increased proapoptotic/prosurvival factor ratio of B-cell leukemia/lymphoma 2 protein associated X protein (Bax) to B-cell leu-

kemia/lymphoma 2 protein (Bcl-2) (Bax/Bcl-2) results in the release of cytochrome c to activate caspase 9 and downstream effectors, including caspase 3, which then leads to cellular apoptosis. It has been reported that active Bax is maintained at the Golgi, rather than mitochondria, in human ES cells (hES) [12], and that hES express elevated levels of multiple proapoptotic Bcl-2 family members, such as Bcl-2-like 11 (Bim), Bcl-2-interacting killer (Bik), and p53-upregulated modulator of apoptosis (Puma) [13]. In contrast, it has also been shown that hES cells acquire responsiveness to tumor necrosis factor-related apoptosis-inducing ligand (Trail) [14].

The DNA methyl-CpG-binding domain (Mbd) protein family consists of DNA methylation readers involved in heterochromatin formation and/or DNA methylation-mediated transcriptional repression [15]. Mbd3 has been originally identified in both mice and humans [16], and it directly regulates the expression of pluripotency genes in ES cells to modulate transcriptional heterogeneity and to maintain ES cell lineage commitment [17]. It has also been reported that the Mbd3/NuRD complex blocks the reprogramming of induced pluripotent stem (iPS) cells [18]. However, there is little information on the role of Mbd3 in mES cell apoptosis. In this study, we evaluated the specific functions of Mbd3 in mES apoptosis using PKCi as well as the expression of several important genes related to mitochondria-mediated apoptosis, including *Bax*, *Bcl-2*, and *Bim*, and death-receptor signaling-mediated apoptosis, including *Fasl*, *Trail,* and downstream effector *caspase 3*. We demonstrated that Mbd3 overexpression induced mES cell apoptosis in the presence of PKCi, and these effects were increased upon removal of PKCi from the mES cell culture. Furthermore, this phenomenon was partially reversed by Mbd3 knockdown.

Materials and methods

Chemicals and reagents

Unless otherwise declared, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animal maintenance, superovulation, and blastocyst collection

Animal experimental protocols were approved by the Animal Care and Use Committees of Nanjing Normal University (NSD-2013-30) and were performed according to the guidelines from the U.S. National Institutes of Health. Female C57BL/6J mice (6-8 weeks old) were intraperitoneally injected with 7.5 IU pregnant mare serum gonadotropin (PMSG; Ningbo Second Hormone Factory, China). After 48 h, the mice were then intraperitoneally injected with 7.5 IU human chorionic gonadotropin (hCG; Ningbo Second Hormone Factory) and mated with males (1:1). Blastocysts were flushed from the uterus on day 3.5 with M2 medium.

De novo derivation of mES

Blastocysts (C57BL/6J) were seeded and co-cultured with mitomycin C-treated mouse embryonic fibroblasts (MEF) in 0.1% gelatin-coated plates (ES-006-B, Millipore, Burlington, MA, USA) with PKCi (5 μM Gö6983, 133053-19-7, Selleck, Houston, TX, USA) and knockout Dulbecco's modified Eagle medium (DMEM; 10829018, Gibco, Gaithersburg, MD, USA) containing 15% knockout serum replacement (KOSR; 10828028, Gibco), 1% penicillin/streptomycin (PS; SV30010, HyClone, Logan, UT, USA), 2 mM glutamine (35050061, Gibco), 1 mM sodium pyruvate (11360088, Gibco), 0.1 mM 2-mercaptoethanol (ES-007-E, Millipore), and 5 μM Gö6983. Seven days after seeding, cell outgrowths were picked and digested into single-cell suspensions with accutase (A1110501, Gibco). The cells were then re-seeded into plates with new MEF feeder cells. Cells were passaged by incubating colonies with accutase, followed by plating into a new 24-well plate with feeder cells at a density of 1×10^3 cells/cm^2 every 3-4 days. Cell counting assay was performed as previously described [19]. Briefly, mES were collected from the 24-well plate (area, 2 cm^2/well) after accutase digestion, cells were counted under an inverted fluorescence microscope, and the total cell number was calculated per well. The collected mES were suspended in cryopreservation medium with 90% fetal bovine serum (FBS; SH30070.03, HyClone) and 10% dimethyl sulfoxide (DMSO; D5879) and stored in liquid nitrogen.

Chimera generation and germline transmission

After superovulation, mating, and blastocyst recovery, as described in detail above [20], 12-15 mES (C57BL/6J) were injected into the blastocoel of each blastocyst (ICR). The injected blastocysts were then surgically transferred

into uterus of pseudopregnant ICR recipient female mice. Pseudopregnancy was achieved by mating with vasectomized males. The recipient females carried the pregnancies to term, and the offspring were weaned at 3 weeks. Chimeras (C57BL/6J mES with ICR embryos) were determined by coat color. Three male chimeras generated from ES cell injection were mated with adult female C57BL/6J mice to test for mES (C57BL/6J) originated germline transmission.

Mbd3 overexpression in mES

The *Mbd3* overexpression plasmids, FUW-Mbd3 (#52356) and control FUW-M2rtTA (#20342), were purchased from Addgene. Lentiviral infection and overexpression were performed as previously described [21]. Briefly, 293T cells were cultured for 2 days in DMEM (C11995500BT, Gibco) supplemented with 10% FBS (v/v) at 37°C in 5% CO_2. Cells at 70-80% confluency were transfected with either FUW-Mbd3 or control FUW-M2rtTA, along with the viral packaging plasmids, psPAX and pMD2.G (5:3:2), using Lipofectamine 2000 reagent (1947415, Invitrogen, Grand Island, NY, USA) at a 1:2 ratio of DNA (g) to Lipofectamine 2000 (μL). The medium was replaced 6 h after infection, and viral supernatants were collected and filtered (0.45-μm pore size; Millipore) 48 h after transfection, respectively. The filtered viral supernatants were used for mES cell infection. For lentiviral transfection, passage 5 mES at 70-80% confluency were infected with the viral supernatants (FUW-Mbd3 or FUW-M2rtTA) supplemented with an equal volume of fresh PKCi medium. After 12 h, the mES were repeatedly infected with the viral supernatants. If necessary, the mES were infected with the viral supernatants up to four times within 48 h.

RNA interference in mES

Lentiviral supernatants containing short hairpin RNA (shRNA) targeting mouse *Mbd3* mRNA (shMbd3) or an shRNA negative control (shNC) were purchased from GenePharma (Shanghai, China). The *shMbd3* target sequence was denoted as GCCTCCTTATCATAGGACAAG, and the shNC sequence was TTCTCCGAACGTGTCACGT. RNA interference was performed according to the manufacturer's instructions. Briefly, after passage 3 mES were cultured for 1-2 days in PKCi medium, lentiviral diluent was added to the mES culture medium, and mES were incubated overnight at 37°C in 5% CO_2. After 24 h, the medium was replaced with PKCi medium, and the cells were incubated for another 24 h at 37°C in 5% CO_2. Infected mES cells were cultured in PKCi removed ES medium for 48 h and collected for further analyses and for Western blot assay, respectively. The shNC infection was defined as knockdown control (-PKCi+shNC).

Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; 000208, EnoGene, Nanjing, China), according to the manufacturer's instructions. Briefly, mES were seeded into a 96-well plate, 10 μL CCK-8 solution was added to each well, and the plate was incubated at 37°C for 1-4 h in the dark. The optical density was measured at 450 nm using an automated microplate reader (BioTek, Winooski, VT, USA). The viability data from the PKCi group were normalized and defined as 1.0 for comparisons among other treatments.

Cell apoptosis assay

Cell apoptosis was measured using the Annexin V-kFluro594 Cell Apoptosis Kit (KAGV109, KeyGEN BioTECH, Nanjing, China), according to the manufacturer's instructions. Briefly, mES were collected and resuspended with binding buffer. Annexin V-kFluro594 reagent was added, and the cells were incubated for 5-15 min at room temperature in the dark. The cells were then washed with Dulbecco's phosphate-buffered saline (DPBS; SH30028.02, HyClone), counterstained with 5 μg/mL Hoechest33342 (BD5011, Bioworld, Nanjing, China), incubated at 30°C for 5 min in the dark, and washed again with DPBS. Apoptotic cells were visualized by red fluorescence, and the nuclei were visualized by blue fluorescence. The cellular apoptosis rate (%) was calculated by dividing the number of apoptotic cells (Annexin V-stained cells; red) by the total number of cells (Hoechst-stained nuclei; blue).

Immunocytochemical staining for pluripotency markers

Immunocytochemical staining was performed for seven pluripotency markers: Nanog (1:200 dilution, Lannuo Biotechnologies, Jiangsu, Chi-

Table 1. Sequences of primers used for qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR Condition	Size (bp)
Mbd3	CAGCCATTGCGAGTGCTCTAC	CTGTCACCATGAAGGCTTTGC	95°C 15 s, Annealing/Extension 60°C 60 s, 40 Cycles	129
Bax	CCGAAATGTTTGCTGACG	AGCCGATCTCGAAGGAAGT	95°C 15 s, Annealing/Extension 60°C 60 s, 40 Cycles	154
Bcl-2	TTCTTTGAGTTCGGTGGGG	CCAGGAGAAATCAAATAGAGGC	95°C 15 s, Annealing/Extension 60°C 60 s, 40 Cycles	195
Bim	TATGGAGAAGGCATTGAC	TGTGGTGATGAACAGAGG	95°C 15 s, Annealing/Extension 60°C 60 s, 40 Cycles	207
Trail	CCTGGAAAGCGACTGAAC	ACCGAAAGTGTCTGTGGC	95°C 15 s, Annealing/Extension 60°C 60 s, 40 Cycles	124
Fasl	GGGTCTACTTACTACCTCAC	CCCTCTTACTTCTCCGTTA	95°C 15 s, Annealing/Extension 60°C 60 s, 40 Cycles	192
caspase 3	ACAGCACCTGGTTACTATTC	CAGTTCTTTCGTGAGCAT	95°C 15 s, Annealing/Extension 60°C 60 s, 40 Cycles	255
GAPDH	GTGGCAAAGTGGAGATTGTTG	CTCCTGGAAGATGGTGATGG	95°C 15 s, Annealing/Extension 60°C 60 s, 40 Cycles	164

na), Oct4 (1:200 dilution, Lannuo Biotechnologies), Sox2 (1:200 dilution, Lannuo Biotechnologies), Klf4 (1:200 dilution, Lannuo Biotechnologies), Rex1 (1:200 dilution, Lannuo Biotechnologies), Fgf4 (1:200 dilution, ab106355, Abcam, Cambridge, UK), and Fgf5 (1:200 dilution, ab88118, Abcam). The mES at passage 7 were washed with DPBS three times and were then fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were then washed again with DPBS three times, permeabilized by incubation with 0.2% Triton X-100 (T8200, Solarbio, Shanghai, China) for 5 min. The cells were incubated with 2% FBS for 30 min at room temperature to block nonspecific binding, subsequently incubated with primary antibodies overnight at 4°C. The next day, cells were washed with DPBS, and incubated with FITC-conjugated secondary antibody (goat anti-rabbit IgG (H+L), 1:300 dilution, AS011, ABclonal, Wuhan, China) for 2 h at room temperature. Afterwards, the cells were washed with DPBS and stained with 100 ng/mL DAPI (BD5010, Bioworld) for 10 min at room temperature in the dark. Finally, cells were washed with DPBS and observed under an inverted fluorescence microscope.

Quantitative PCR (qPCR)

Total RNA was extracted from mES samples with Trizol reagent (T9424). Reverse transcription reactions were performed with 1 μg RNA using the HiScript II Reverse Transcriptase (R223-01, Vazyme, Nanjing, China). Complementary DNA (cDNA) was used as the template, and the 2xSYBR Green Fast qPCR Mix with High Rox (RM21206, ABclonal) was used for the qPCR reaction. The qPCR primers are shown in **Table 1**. Individual gene expression was normalized to *Gapdh* expression, and data from the PKCi group were defined as 1.0 for all gene expression levels.

Western blot

Total proteins were collected from mES using lysis buffer and were quantified using the BCA Kit (GK5012, Beyotime Biotechnology, Wuhan, China). Western blot analysis was performed as previously described [22]. Briefly, 6 μg of total protein was loaded into each well of a 12% gel for SDS-PAGE. After electrophoresis, the separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (03010040001, Roche, Basel, Switzerland) by electrotransfer. The membranes were blocked with 5% non-fat powdered milk (A600669, Sangon Biotech, Shanghai, China) for 1 h, washed with tris-buffered saline with Tween 20 (9005-64-5, Sangon Biotech) (TBST), and then incubated with anti-Mbd3 (1:1,000 dilution, A2251, Lot 0046180101, ABclonal), anti-Bax (1:1,000 dilution, A19684, Lot 4000000164, ABclonal), or anti-Bcl-2 (1:1,000 dilution, A19693, Lot 4000000173, ABclonal) antibodies overnight at 4°C. The next day, the membranes were washed with TBST and incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:5,000 dilution, BS13278, Bioworld) for 1 h. After washing with TBST, the membranes were processed using an enhanced chemiluminescence reagent (E411-04, Vazyme), and the protein bands were visualized using a LAS-4000 imager (Tanon, Shanghai, China). β-actin (1:1,000 dilution, AC026, Lot 9100026001, ABclonal) was used as an internal control. The Mbd3, Bax, and Bcl-2 protein expression levels were normalized to those of β-actin. The data from the PKCi group for all three proteins were defined as 1.0 and were used for comparisons with other treatments.

Statistical analysis

Every experiment was repeated at least three times. Data were analyzed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) and were

provided as the mean ± standard deviation. Statistical comparisons were made by analysis of variance (ANOVA). Values of $P<0.05$ were considered significant (*), and values of $P<0.01$ were considered extremely significant (**). Significant difference was indicated by a, b, and c among the groups ($P<0.05$).

Results

Germline transmission was achieved using mES cultured in PKCi medium

Domed-shaped mES were cultured in PKCi medium and maintained in an undifferentiated state for seven consecutive passages. The mES expressed core stem cell pluripotency markers, Nanog (**Figure 1A1-A4**), Oct4 (**Figure 1B1-B4**), and Sox2 (**Figure 1C1-C4**), as well as naïve cell markers, Klf4 (**Figure 1D1-D4**), Rex1 (**Figure 1E1-E4**), and Fgf4 (**Figure 1F1-F4**). However, the mES did not express the primed cell marker, Fgf5 (**Figure 1G1-G4**).

Third-passage mES (XY) derived in PKCi medium were transferred into recipient blastocysts to establish germline transmission. After ES injection, a total of 207 mES (C57BL/6J) injected embryos (ICR) were transferred into nine recipient female ICR mice. The recipient mice gave birth to a total of 38 (18.4% birth rate) offspring, consisting of 16 colored chimeras (42.1%). Three male chimera founders were mated with C57BL/6J female mice to examine the germline transmission. One chimera founder produced homozygous offspring, giving a transmission rate of 33.3%.

Overexpression of Mbd3 induced mES cell apoptosis

To understand the role of Mbd3 in mES cell apoptosis, Mbd3 overexpression using the FUW-Mbd3 plasmid was performed in mES cultured in PKCi medium. Compared with the PKCi and control groups, both the Mbd3 mRNA (**Figure 2A**) and protein (**Figure 2B**) expression levels were significantly increased ($P<0.01$). Mbd3 overexpression dramatically reduced the total cell number (0.23×10^4) compared with the PKCi (0.85×10^4) and control (FUW-M2rtTA; 0.79×10^4) groups ($P<0.01$; **Figure 2C**). Additionally, Mbd3 overexpression resulted in a significant reduction in cell viability 0.55 ($P<0.01$; **Figure 2D**) compared with the PKCi and control groups (100%) and a significant increase in the cell apoptosis rate (54.8%) compared with those of the PKCi (0.9%) and control (1.1%) groups ($P<0.01$; **Figure 2E**).

Western blot analysis indicated that the relative Bcl-2 protein expression levels were dramatically decreased (0.3 Bcl-2/β-actin intensity ratio) in response to Mbd3 overexpression compared with the PKCi (1.0) and control (1.0) groups ($P<0.01$), whereas the Bax protein expression levels were similar among the three groups (**Figure 2F**). In turn, Mbd3 overexpression resulted in a 3-fold increase in the Bax/Bcl-2 ratio. Moreover, Mbd3 overexpression significantly increased the relative mRNA expression of proapoptotic genes, including *Bax* (from 1.0 for the PKCi and control groups to 3.1) and *Bim* (from 1.0 for the PKCi and control groups to 2.6), as well as death-receptor ligand genes, including *Trail* (from 1.0 for the PKCi group and 1.1 for the control group to 6.0), *FasI* (from 1.0 for the PKCi and control groups to 3.4), and *caspase 3* (from 1.0 for the PKCi and control groups to 2.1), whereas the expression of prosurvival *Bcl-2* mRNA was decreased (from 1.0 for the PKCi and control groups to 0.5) (**Figure 2G**). Similar RNA expression levels were observed for all six genes between the control and PKCi groups.

PKCi removal resulted in increased Mbd3 expression and cell apoptosis, which were partially reversed by Mbd3 knockdown

When PKC inhibitor Gö6983 was removed from the mES cell culture medium for 48 h, the Mbd3 mRNA (**Figure 3A**) and protein (**Figure 3B**) levels increased ($P<0.05$). This also caused a reduction in the total cell number from 0.9×10^4 cells to 0.1×10^4 cells (**Figure 3C**) as well as the cell viability from 1 to 0.29 (**Figure 3D**; $P<0.05$). However, knockdown of *Mbd3* mRNA in mES under culture without PKCi addition reduced the Mbd3 mRNA level from 2.6 to 1.6 and protein level from 1.8 to 1.3, respectively, compared with the PKCi control group (normalized as 1.0) ($P<0.05$), but these levels were still higher than those of the PKCi group (**Figure 3A** and **3B**). As a result, the total number of mES increased from 0.1×10^4 to 0.4×10^4 cells (**Figure 3C**; $P<0.05$), and the cell viability was partially recovered from 0.29 to 0.48 (**Figure 3D**; $P<0.05$). Moreover, *Mbd3* mRNA knockdown

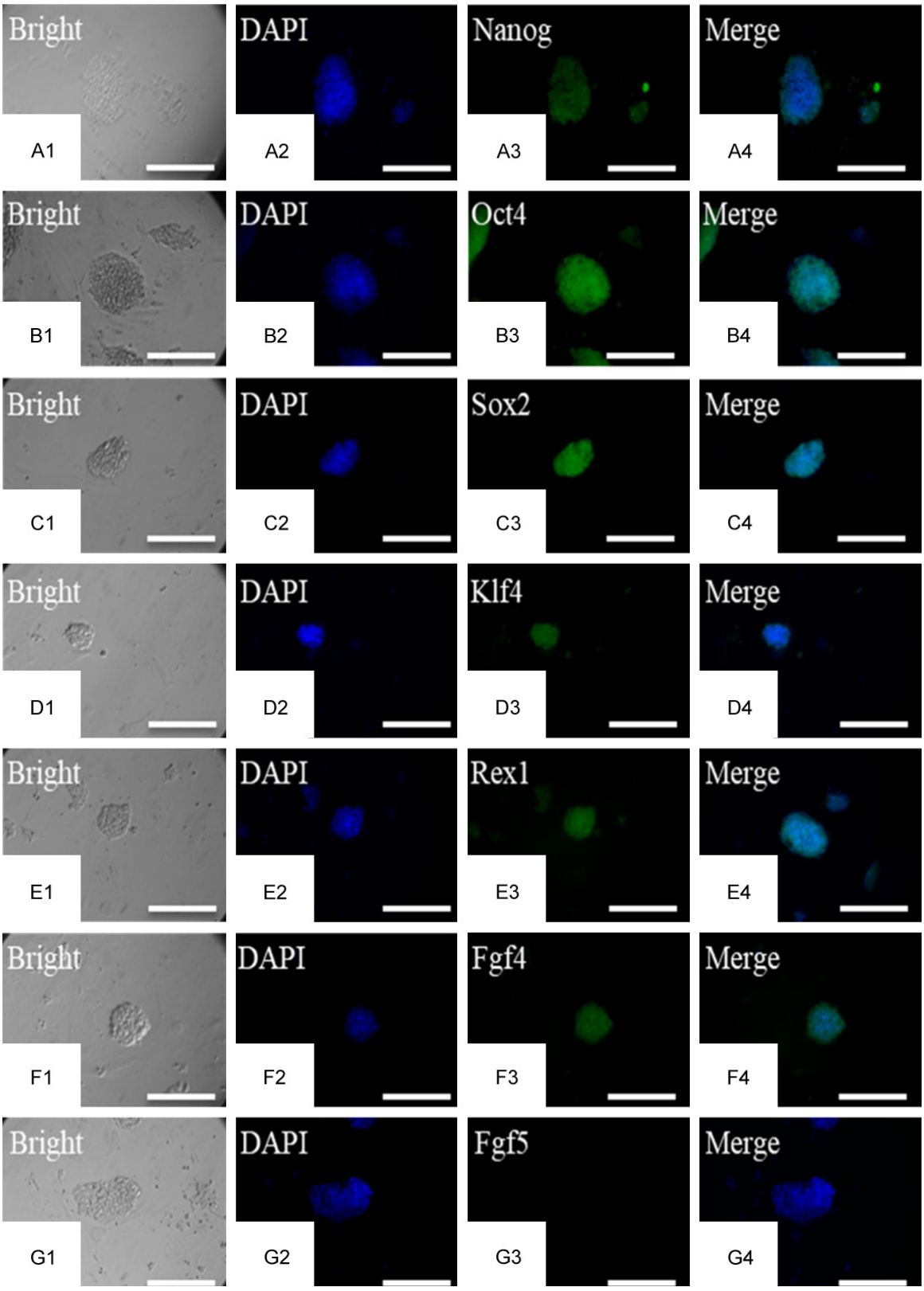
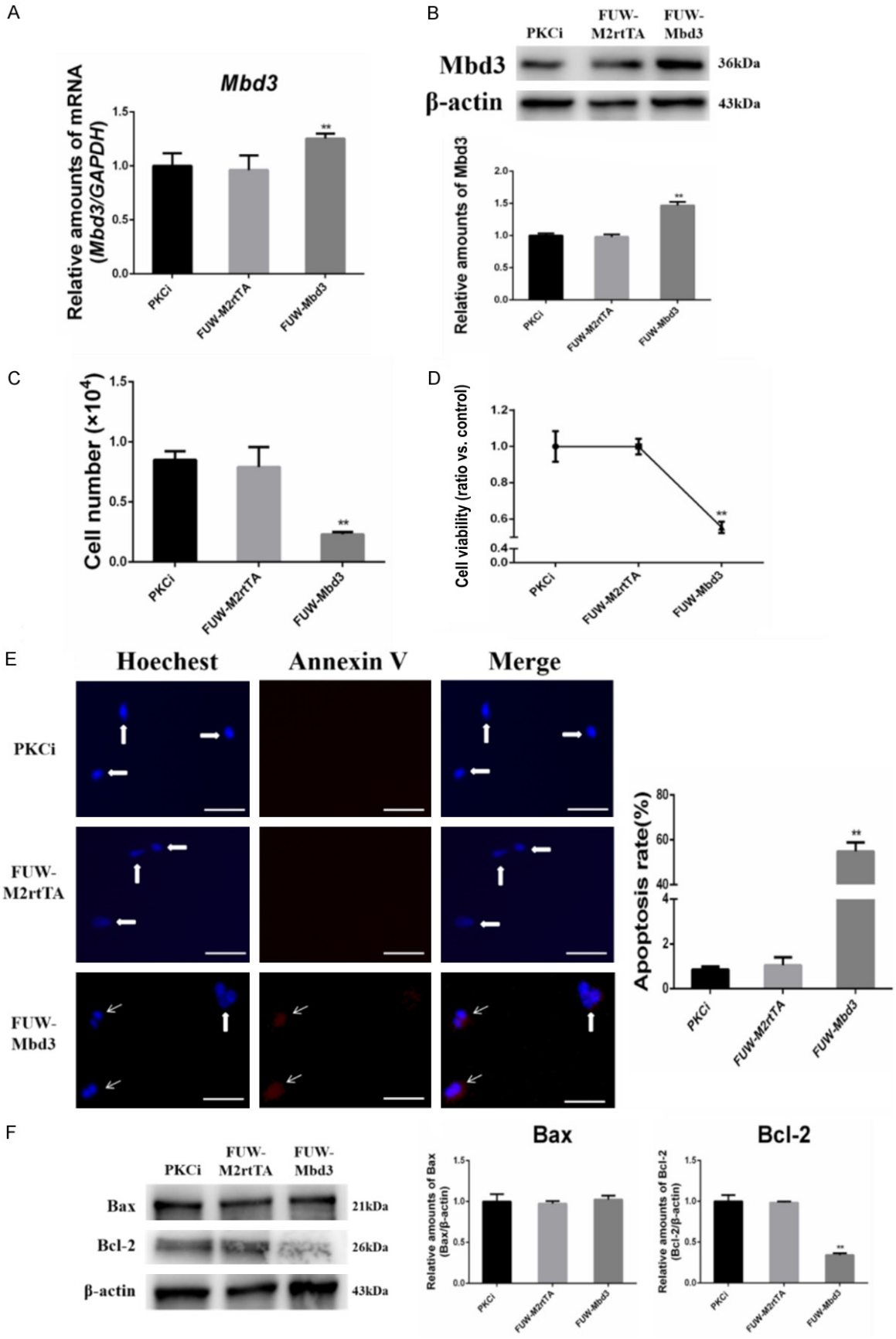


Figure 1. Immunochemistry of mES derived and maintained in PKCi medium. Immunostaining indicated that core pluripotency markers, Nanog (A1-A4), Oct4 (B1-B4), and Sox2 (C1-C4), naïve ES cell makers, Klf4 (D1-D4), Rex1 (E1-E4), and Fgf4 (F1-F4), but not primed cell marker, Fgf5 (G1-G4), were expressed in seventh-passage mES maintained in PKCi medium. Scale bar =400 μm.


A
Mbd3
Relative amounts of mRNA (Mbd3/GAPDH)
PKCi
FUW-M2rtTA
FUW-Mbd3
B
PKCi
FUW-M2rtTA
FUW-Mbd3
Mbd3
36kDa
β-actin
43kDa
Relative amounts of Mbd3
C
Cell number (×10⁴)
D
Cell viability (ratio vs. control)
E
Hoechest
Annexin V
Merge
PKCi
FUW-M2rtTA
FUW-Mbd3
Apoptosis rate(%)
F
PKCi
FUW-M2rtTA
FUW-Mbd3
Bax
21kDa
Bcl-2
26kDa
β-actin
43kDa
Bax
Relative amounts of Bax (Bax/β-actin)
Bcl-2
Relative amounts of Bcl-2 (Bcl-2/β-actin)

G

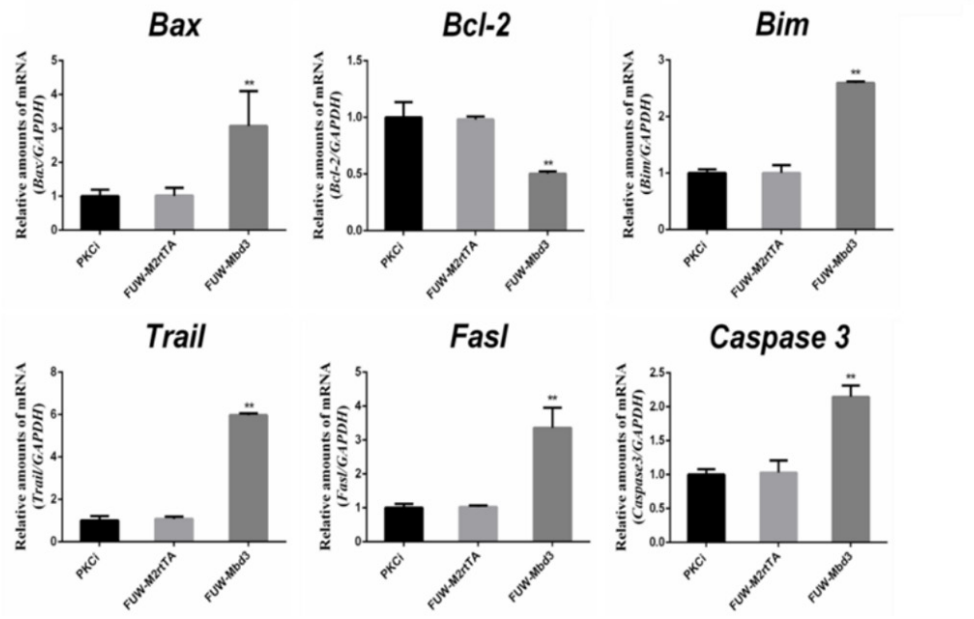
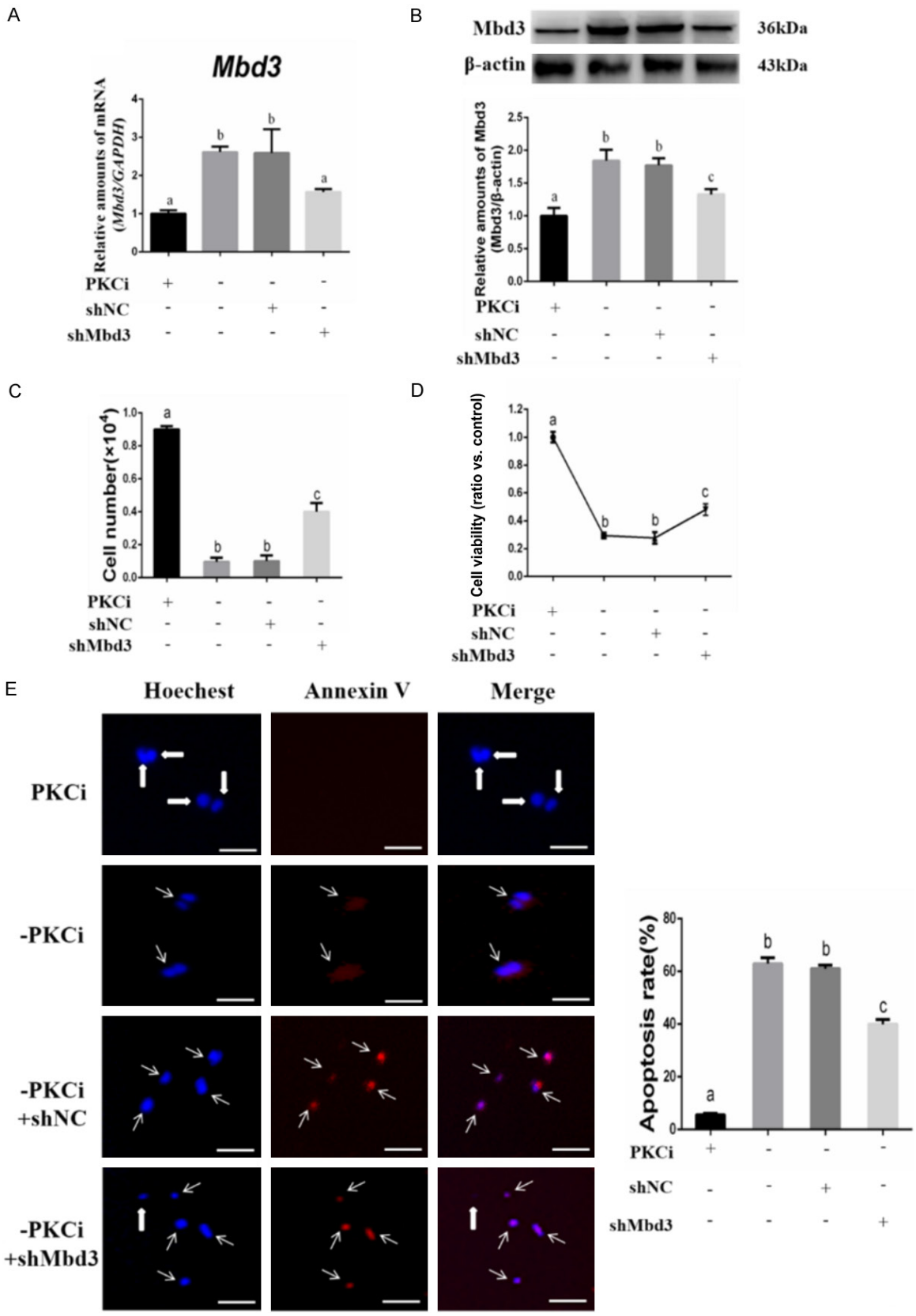


Figure 2. Mbd3 overexpression induced cellular apoptosis. A. Overexpression of *Mbd3* RNA levels was achieved by transfection with the FUW-Mbd3 plasmid in fifth-passage mES. The FUW-M2rtTA plasmid and the PKCi group were used as controls. B. Mbd3 protein expression was evaluated by Western blot in fifth-passage mES (upper panel). Quantitative density analysis showed that Mbd3 overexpression resulted in its increased protein levels compared with the PKCi control (lower panel). C. Mbd3 overexpression significantly reduced the total number of fifth-passage mES. D. CCK8 assay revealed that Mbd3 overexpression decreased mES cell viability. E. Apoptosis was evaluated by Annexin V staining in fifth-passage mES. The thick arrow indicates cell nuclei (blue), and the thin arrow indicates apoptotic cells (red). Scale bar =100 μm (left panel). The apoptosis rate was significantly increased with Mbd3 overexpression (right panel). F. Bax, Bcl-2, and β-actin protein levels were evaluated by Western blot in fifth-passage mES (left panel). Quantitative density analysis showed similar levels of Bax in all three groups (middle panel) and significantly reduced Bcl-2 levels with Mbd3 overexpression (right panel). G. Mbd3 overexpression increased the expression of *Bax*, *Bim*, *Trail*, *Fasl,* and *caspase 3* RNA, but reduced the expression of *Bcl-2* RNA, in fifth-passage mES. The data are represented as mean ± SD (n=3). **P*<0.05, ***P*<0.01.

without PKCi did not fully recover the total cell number or cell viability of mES to the levels observed with PKCi (**Figure 3A-D**; *P*<0.05). When PKCi was removed from the culture medium, the rate of apoptosis increased from 5.7% to 63.0% (**Figure 3E**; *P*<0.05), whereas the knockdown control group exhibited a similar level (61.1%) as that of the control group without PKCi (*P*>0.05). In contrast, knockdown Mbd3 reduced the apoptosis rate to 40.0%, which was lower than that of the knockdown control group (-PKCi+shNC) as well as the control group without PKCi (*P*<0.05); however, this level was still higher in the PKCi group (**Figure 3E**; *P*<0.05).

Western blot analysis showed that PKCi removal resulted in elevated Bax protein levels (from 1.0 to 1.6) with reduced Bcl-2 protein levels (from 1.0 to 0.7) in mES (*P*<0.05). In addition, the knockdown control group (-PKCi+shNC) exhibited similar Bax and Bcl-2 levels to those of the group without PKCi (**Figure 3F**). Mbd3 knockdown significantly reduced Bax levels to 1.3 compared with the knockdown control group (1.6) and the group without PKCi (1.6) (*P*<0.05), but the Bax levels were still higher in the PKCi group (1.0) (*P*<0.05; **Figure 3F**). In contrast, the Bcl-2 protein levels (0.7) with Mbd3 knockdown were similar to those of the knockdown control group and the group without PKCi (*P*>0.05); however, the levels were not as high as the levels of the PKCi group (1.0) (*P*<0.05; **Figure 3F**).

Results from qPCR indicated that PKCi removal increased *Bax* (from 1.0 to 3.1), *Bim* (from 1.0 to 3.5), *Trail* (from 1.0 to 4.1), *Fasl* (from 1.0 to 2.5), and *caspase 3* (from 1.0 to 5.6) RNA expression levels (*P*<0.05), but reduced *Bcl-2* levels (from 1.0 to 0.7) (**Figure 3G**). However, compared with knockdown control group (-PK-


A
Mbd3
Relative amounts of mRNA (Mbd3/GAPDH)
PKCi
shNC
shMbd3
B
Mbd3
β-actin
36kDa
43kDa
Relative amounts of Mbd3 (Mbd3/β-actin)
C
Cell number(×10⁴)
D
Cell viability (ratio vs. control)
E
Hoechest
Annexin V
Merge
PKCi
-PKCi
-PKCi +shNC
-PKCi +shMbd3
Apoptosis rate(%)

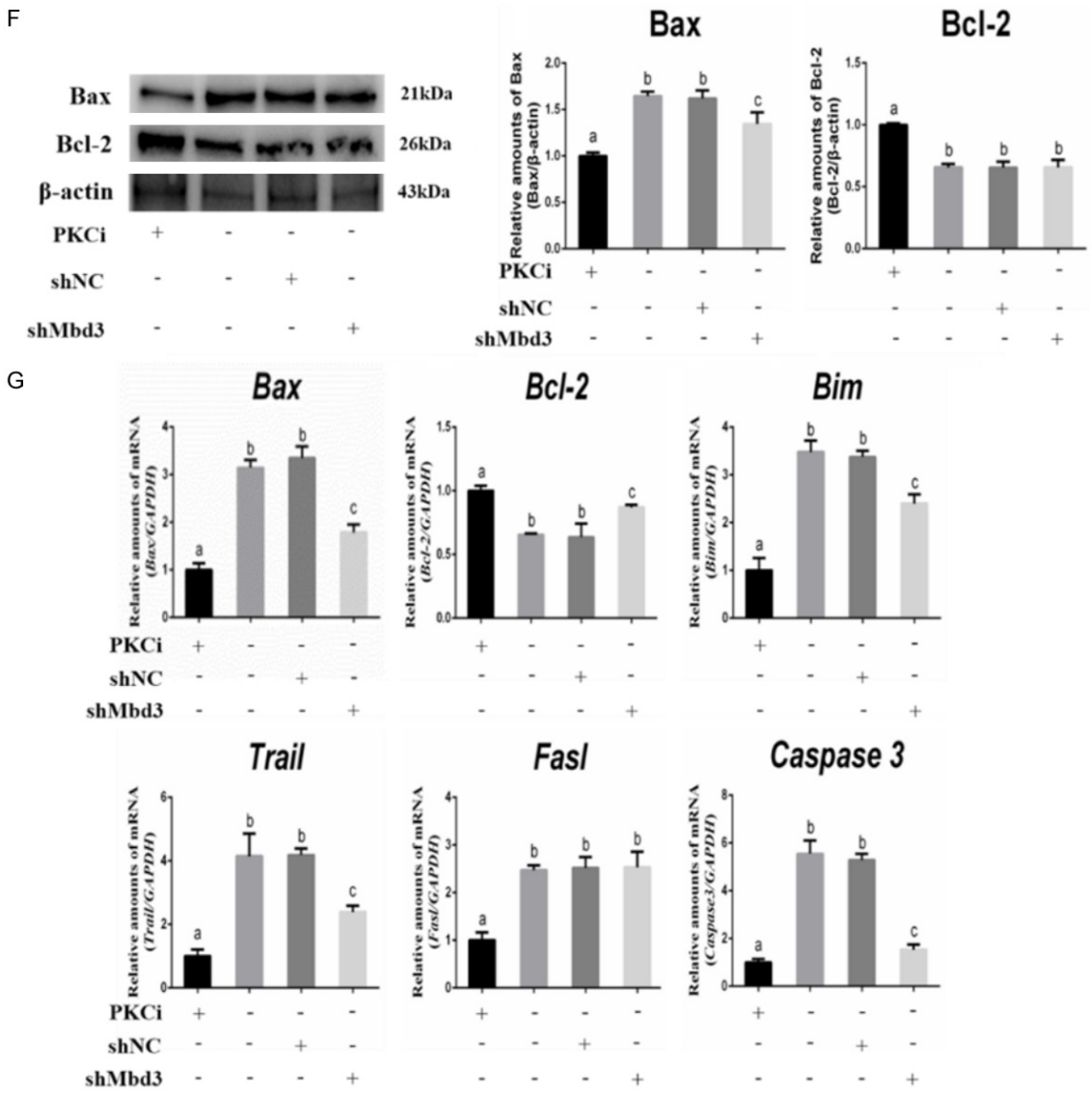


Figure 3. Mbd3 knockdown partially reversed the cellular apoptosis induced by PKCi removal. A. *Mbd3* RNA expression levels were increased in third-passage mES when PKCi was removed for 48 h from the culture medium, but they were only partially decreased with Mbd3 knockdown. B. Mbd3 protein levels were assessed by Western blot in third-passage mES (upper panel). Quantitative density analysis showed a significant increase in Mbd3 upon PKCi removal, and a partial reduction was observed with Mbd3 knockdown. C. The total number of third-passage mES was dramatically decreased upon PKCi removal and increased with Mbd3 knockdown, but it was still lower than that of the PKCi group. D. Cell viablility was improved by Mbd3 knockdown. E. Cellular apoptosis was assessed by Annexin V staining in third-passage mES. The thick arrow indicates cell nuclei (blue), and the thin arrow indicates apoptotic cells (red). Scale bar =100 μm (left panel). The apoptosis rate was significantly increased upon PKCi removal, but it was partially reduced with Mbd3 knockdown (right panel). F. Bax, Bcl-2, and β-actin levels were evaluated by Western blot in third-passage mES with Mbd3 knockdown (left panel). Quantitative density analysis showed increased levels of Bax after PKCi removal, but only partially reduced levels were observed with Mbd3 knockdown (middle panel). Bcl-2 expression was reduced upon PKCi removal, and similar levels were observed with Mbd3 knockdown (right panel). G. PKCi removal resulted in increased expression levels of *Bax*, *Bim*, *Trail*, *FasI,* and *caspase 3* RNA, with reduced *Bcl-2* levels, in third-passage mES. Mbd3 knockdown resulted in partially reduced *Bax*, *Bim*, *Trail,* and *caspase 3* RNA levels, unchanged *FasI* levels, and increased *Bcl-2* levels. The data are represented as mean ± SD (n=3). The letters a, b, c indicate significant differences among the groups ($P<0.05$).

Ci+shNC), Mbd3 knockdown without PKCi resulted in decreased *Bax* (from 3.3 to 1.8), *Bim* (from 3.4 to 2.4), *Trail* (from 4.2 to 2.4), and *caspase 3* (from 5.3 to 1.5) RNA expr-

ession levels ($P<0.05$), but the *Fasl* levels remained the same (from 2.5 to 2.5; $P>0.05$). Additionally, Mbd3 knockdown without PKCi increased *Bcl-2* RNA expression levels (from 0.6 to 0.9; $P<0.05$), but these levels were still lower than those of the PKCi group (1.0; $P<0.05$). The knockdown control (-PKCi+shNC) did not exhibit any changes in the RNA expression levels for all six genes compared with those of the group without PKCi (**Figure 3G**).

Discussion

In this study, we demonstrated Mbd3 as an important regulator of apoptosis in mES using PKCi. The mES maintained an undifferentiated state in the presence of PKCi in the culture medium compared with the conventional 2iL method [6]. Mbd3 overexpression with PKCi induced apoptosis in the mES. Mbd3 expression at both the RNA and protein levels was dramatically upregulated upon PKCi removal. This subsequently resulted in the elevated expression of key proapoptotic genes, including *Bax* and *Bim*, as well as death-receptor ligand genes, including *Trail* and *Fasl*, and also *caspase 3*. Additionally, the reduced expression of prosurvival gene *Bcl-2* was also observed. These changes resulted in an increased cellular apoptosis rate and a decreased total cell number and viability. In contrast, Mbd3 knockdown by RNA interference partially reversed these effects due to the removal of PKCi in mES. Therefore, our results strongly indicate that Mbd3 plays a pivotal role in mES cell apoptosis.

Cellular apoptosis is a highly complex and sophisticated process that involves two main apoptotic pathways, death-receptor signaling and mitochondria-mediated apoptosis. The extrinsic (death-receptor signaling) and intrinsic (mitochondria-mediated apoptosis) pathways trigger apoptosis by activating different caspase cascades and by converging to the executor of apoptosis, caspase 3 [23], subsequently leading to a series of cellular responses, such as DNA fragmentation, degradation of nuclear and cytoskeletal proteins, crosslinking of proteins, cell shrinkage, and membrane blebbing. The extrinsic signaling pathway initiates apoptosis by transmembrane receptor-mediated interactions between ligands and homologous death receptors, such as Fasl/CD95 and Trail/DR4 [11, 24-27]. Fasl and Trail ligand binding to their respective receptors results in the recruitment of adapter protein Fas-associated protein with death domain (FADD) [28, 29]. Subsequently, an association with procaspase 8 via the dimerization of the death effector domain and the formation of a death-inducing signaling complex [9] results in the autocatalytic activation of procaspase 8. Activated caspase 8 then induces apoptosis by two parallel cascades. One cascade activates caspase 8 either by directly cleaving and activating caspase 3 or by cleaving BH3-interacting domain death agonist (Bid) [30], which is a proapoptotic protein of the Bcl-2 family. Truncated Bid (tBid) then induces the mitochondrial translocation of Bcl-2 homologous antagonist/killer (Bak), which induces cytochrome c release and finally activates caspase 9 and caspase 3. The Bcl-2 family is known to modulate cellular apoptosis through the mitochondria-mediated pathway [31]. Bim, Bax, Bik, Bcl-2 agonist of cell death (Bad), Bak, and Harakiri Bcl-2-interacting protein (Hrk) [32] are all proapoptotic proteins, whereas Bcl-2 is a prosurvival protein. Proapoptotic proteins promote cellular apoptosis, whereas prosurvival proteins inhibit it [32-34]. Bim belongs to the Bcl-2 family and encodes a BH3-only protein and induces apoptosis through the mitochondria-mediated pathway. In viable cells, Bax resides as its monomeric form in the cytosol or membrane, whereas prosurvival proteins Bcl-2 and Bcl-xL exist in the outer mitochondrial membrane and inhibit the release of cytochrome c. The intrinsic pathway is activated by several stimuli, including the translocation of monomeric Bad from the cytosol to mitochondria, where it forms a proapoptotic complex with Bcl-xL. The inner mitochondrial membrane is then destroyed, resulting in the opening of the mitochondrial permeability transition pore and the loss of the mitochondrial membrane potential, which is accelerated by the opening of mitochondrial voltage-dependent anion channels. The open anion channels allow cytochrome c to pass, which then binds and activates apoptotic peptidase activating factor 1 (Apaf1) and caspase 9, eventually activating effector caspase 3 and resulting in cellular apoptosis [35, 36]. Overall, during this regulatory process, the balance between Bax/Bcl-2 determines the apoptotic cell fate [37]. It has been reported that FoxO1 upregulation due to oxidative stress causes an increase in the expression of downstream apoptosis-related genes, including *Fasl*, *Trail*, *Bim*, and *caspase 3*, leading to apoptosis in granulosa cells [23].

Mbd3 is located in the nucleus, where it functions as a repressor by recruiting additional chromatin-remodeling proteins and by reading and binding to methylated DNA and its associated histones [38]. It plays an important role in chromatin assembly, gene stability, and in particular, gene silencing [39-41]. Mbd3 regulates cell cycle progression and cell death by mediating important cell cycle genes, including cyclin B1 (*Ccnb1)*, 1-phosphatidylinositol 4-kinase (*Plk1*), and survivin in cancer cells [42]. However, there is little information on the role of Mbd3 in the regulation of key proapoptotic genes, such as *Fasl*, *Trail*, *Bim*, *Bax*, and effector *caspase 3*, or prosurvival genes, such as *Bcl-2,* in mES. In this study, naïve-state mES were cultured in the presence of PKCi, and chimera generation with these cells elicited germline transmission in F1 progeny. In PKCi medium, the rate of apoptosis of the mES was very low. Mbd3 overexpression resulted in an increase in both its RNA and protein expression levels. Furthermore, Bcl-2 protein levels were significantly reduced, whereas Bax levels remained consistent, resulting in a 3-fold increase in the Bax/Bcl-2 ratio. Mbd3 overexpression also resulted in the increased expression of *Bax*, *Bim*, *Trail*, *Fasl,* and *caspase 3* mRNA; however, the expression of *Bcl-2* was decreased. It has been reported that cisplatin upregulates the expression levels of Trail, initiator caspase 8, and effector caspase 3, leading to apoptosis in hES [14]. The upregulation of p53, caspase 9, and caspase 3 and an increased Bax/Bcl-2 ratio have also been observed during apoptosis induced by zearalenone (ZEN) in mES and hES [43]. Neural stem cells may possess direct anti-glioma properties via the downregulation of caspase 3 and p53 [44]. During apoptosis, activated Bax is translocated from the Golgi to mitochondria in a p53-dependent manner in hES [12], and mES expressing mutations of the Abl nuclear localization signals (AblμNLS) exhibit delayed Bax activation, reduced cytochrome c release, and decreased caspase 9 activity [45].

We found that apoptosis was accompanied by elevated levels of *Bax*, *Bim*, *Trail*, *Fasl* and *caspase 3,* along with reduced levels of *Bcl-2* in response to Mbd3 overexpression. In contrast, when PKCi was removed from the culture medium, a dramatic increase in both the RNA and protein levels of Mbd3 occurred. Along with this increase, the Bax levels also increased to 1.5, whereas the Bcl-2 levels decreased to 0.7, resulting in a 2.14-fold increase in the Bax/Bcl-2 ratio. As a result, a significant decrease in the total cell number and viability occurred, along with a significant increase in the rate of apoptosis. Inhibition of PKCζ/NF-κB signaling has been shown to inhibit the microRNA-21/microRNA-29 axis, thus maintaining ES cell-specific epigenetic modifications and supporting ES cell self-renewal [6, 7]. It is reasonable to consider that PKCi removal may increase the microRNA-21/microRNA-29 levels, thus inhibiting the translation of some pluripotency genes. However, PKCi removal did not inhibit Mbd3 translation, of which the inherent mechanism remains unknown. Therefore, we assume that there may be the other mechanisms regulating Mbd3 expression at both the transcriptional and translational levels. As expected, the increased Mbd3 expression that occurred in response to PKCi removal caused a significant increase in the RNA expression levels of five key proapoptotic genes, *Bax*, *Bim*, *Trail*, *Fasl,* and *caspase 3*, but a decrease in the expression levels of prosurvival *Bcl-2*. Overall, our study demonstrated that Mbd3 overexpression, either by viral transfection or by PKCi removal, resulted in these gene expression changes. Because Mbd3 is a well-known repressor that directly binds to DNA to repress gene transcription, we hypothesize that *Bcl-2* transcription may be regulated by the direct binding of Mbd3 protein to its regulatory promotor region, thus resulting in its decreased expression upon increased Mbd3 expression. In contrast, Mbd3 indirectly upregulates the expression of *Bax*, *Bim*, *Trail*, *Fasl,* and *caspase 3* by binding to and inhibiting their transcription factor repressors/corepressors. This regulatory mechanism of Mbd3 requires further investigation.

Interestingly, Mbd3 knockdown by RNA interference without PKCi did not alter the Bcl-2 protein levels in mES. However, the Bax protein levels were decreased, resulting in a reduced Bax/Bcl-2 ratio. As a result, the mRNA expression levels of *Bax*, *Bim*, *Trail*, *Fasl,* and *caspase 3* were reduced, but the *Bcl-2* expression levels were increased compared with those of the control group without PKCi. It has been shown that Bcl-2 overexpression improves growth rates, increases clonogenicity, and reduc-

es apoptosis in hES [46]. In our study, although Mbd3 knockdown did not result in increased Bcl-2 protein levels, the decreased Bax/Bcl-2 ratio improved the cell number and viability and reduced the apoptosis rate of the mES compared with the group without PKCi. These results support that Mbd3 regulates apoptosis-related gene expression and apoptosis in mES.

In summary, we found that Mbd3 upregulation decreased the total cell number and viability and increased the apoptosis rate of mES. Additionally, it increased the expression of *Bim*, *Trail*, *Fasl*, and *caspase 3*, but reduced the expression *Bcl-2* RNA, resulting in an increased Bax/Bcl-2 ratio. These effects were partially reversed by Mbd3 knockdown. All of these findings support Mbd3 as a pivotal regulator of apoptosis in mES.

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Disclosure of conflict of interest

None.

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