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c-Jun NH_2-Terminal Kinase Is Required for Lineage-Specific Differentiation but Not Stem Cell Self-Renewal▽†

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The c-Jun NH_2-terminal kinase (JNK) is implicated in proliferation. Mice with a deficiency of either the *Jnk1* or the *Jnk2* genes are viable, but a compound deficiency of both *Jnk1* and *Jnk2* causes early embryonic lethality. Studies using conditional gene ablation and chemical genetic approaches demonstrate that the combined loss of JNK1 and JNK2 protein kinase function results in rapid senescence. To test whether this role of JNK was required for stem cell proliferation, we isolated embryonic stem (ES) cells from wild-type and JNK-deficient mice. We found that *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells underwent self-renewal, but these cells proliferated more rapidly than wild-type ES cells and exhibited major defects in lineage-specific differentiation. Together, these data demonstrate that JNK is not required for proliferation or self-renewal of ES cells, but JNK plays a key role in the differentiation of ES cells.

The c-Jun NH_2-terminal kinase (JNK) is a member of the mitogen-activated protein (MAP) kinase group of signaling proteins. JNK is encoded by two ubiquitously expressed genes (*Jnk1* and *Jnk2*) and by a third gene (*Jnk3*) that is selectively expressed in neurons (14). Gene disruption studies demonstrate that mice without *Jnk1* or *Jnk2* are viable, but compound deficiency of both *Jnk1* and *Jnk2* causes early embryonic lethality (14). Murine embryonic fibroblasts (MEFs) isolated from *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ mice exhibit a severe growth retardation phenotype (54). The markedly reduced growth of *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ MEFs is consistent with the finding that JNK is critically required for the regulation of AP1-dependent gene expression (56) that is implicated in cellular proliferation (26). Thus, *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ MEFs express low levels of AP1 proteins (e.g., c-Jun and JunD) and exhibit marked defects in AP1 target gene expression (34, 56). This loss of AP1 function is mediated, in part, by reduced phosphorylation of the activation domain of Jun family proteins and ATF2 (56).

More recent studies using a conditional gene ablation strategy have demonstrated that compound JNK deficiency causes rapid senescence (12). This conclusion was confirmed by using chemical genetic analysis with MEFs isolated from mice with a germ line mutation that sensitizes JNK to inhibition by a predesigned small-molecule drug (12, 25). This form of senescence was found to be p53 dependent (12) and resembles the p53-dependent senescence of c-*Jun*$^{-/-}$ MEFs (49). These data indicate that JNK plays a critical role in cellular proliferation. Indeed, it is possible that the p53-dependent senescence observed in JNK-deficient cells may contribute to aging. This is because altered p53 function is established to be an important determinant of early aging (36, 55). Importantly, this role of p53 in aging appears to be distinct from p53-mediated tumor suppression and DNA damage responses (21, 39, 43).

One aspect of the aging process is a reduction in the regenerative capacity of stem cells (50). Indeed, it has been established that altered p53 activity associated with aging causes decreased stem cell function (8, 18, 42) and that disruption of the p53 pathway can increase stem cell function (1). Since JNK can influence p53-dependent senescence (12), these data indicate that JNK may be important for stem cell proliferation and self-renewal potential.

Embryonic stem (ES) cells proliferate and are capable of both self-renewal and differentiation to multiple cell types. Indeed, murine ES cells can differentiate to create all tissues within a mouse. The profound growth retardation and rapid p53-dependent senescence of *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ MEFs (12) suggests that JNK may play a critical role in the normal function of ES cells, including self-renewal and differentiation potential. The purpose of the present study was to test this hypothesis. Our approach was to isolate ES cells from wild-type and JNK-deficient mice. We demonstrate that JNK is not required for self-renewal or the proliferation of ES cells. However, JNK is required for ES cell differentiation.

MATERIALS AND METHODS

Mouse studies. *Jnk1*$^{-/-}$ mice (16) and *Jnk2*$^{-/-}$ mice (60) on a C57BL/6J genetic background were described previously. C57BL/6J mice and C57BL/6J-*scid* (B6.CB17-prkdcscid/SzJ) mice were obtained from the Jackson Laboratories. These mice were housed in a facility that is accredited by the American Association for Laboratory Animal Care and the studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Genotype analysis. *Jnk1* and *Jnk2* genotypes were examined by PCR analysis of genomic DNA (16, 60). Sex determination of ES cells was performed by PCR amplification of genomic DNA to detect the presence of X and Y chromosomes (46).

ES cell culture. Blastocysts (embryonic day 3.5 [E3.5]) were isolated (47) and transferred to 24-well tissue culture dishes with a feeder cell layer of primary mouse embryo fibroblasts (MEFs) inactivated with mitomycin C (Sigma) in Dulbecco modified Eagle medium (DMEM; Invitrogen), 15% fetal bovine serum

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TABLE 1. Amplimers used in this study

Gene	Amplimer pair sequences (5′–3′)	
	Amplimer 1	Amplimer 2
Anf	GGGGGTAGGATTGACAGGAT	CAGAGTGGGAGAGGCAAGAC
Apoa2	CACTGCTGGTAACCATCTG	TCGAGGTTCATTAAACTGCT
BMHC	CTACAGGCCTGGGCTTACCT	TCTCCTTCTCAGACTTCCGC
Bmp4	GTTTCCTCTTCAACCTCAGC	TATACGGTGGAAGCCCTGTT
Brachyury	CTCACCAACAAGCTCAATGG	ACAGCTATGAACTGGGTCTC
Emx2	AGCTTTTAAGGCTAGAGCAC	CCTGAGTTTCCGTAAGACTG
Flk1	GGCGGTGGTGACAGTATCTT	CTCGGTGATGTACACGATGC
Gata4	TGTCAATTGTGGGGCCATGTC	GTGCCCCAGCCTTTTACTTTG
Hnf1	GAAAGCAACGGGAGATCCTCCGAC	TAGGCATCCATGGCCAGCTTCTGC
Hnf3	TGGTCACTGGGGACAAGGGAA	GCAACAACAGCAATAGAGAAC
Keratin17	GTAGTGCTCTTGGGAGCAACAG	AGAACCAGTCTTCGGCATCCTT
MEF2c	ACTGGGAAACCCCAATCTTC	ATCAGACCGCCTGTGTTACC
Mlv2v	AAAGAGGCTCCAGGTCCAAT	CCTCTCTGCTTGTGTGGTCA
Nkx2.5	TGCAGAAGGCAGTGGAGCTGGACAAGCC	TTGCACTTGTAGCGACGGTTCTGGAACCAG
Nodal	TGGAGCGCATTTGGATGGAGAC	TACTGCTTGGGGTAGATGATCC
Otx1	TTCACGCGCTCACAGCTGGA	ACCAAACCTGGACTCTGGAC
Pax2	TGCCGAATACAAGCGACAGAAC	TGGAAGACATCGGGATAGGAAG
Sox17	GCCAAAGACGAACGCAAGCGG	TCATGCGCTTCACCTGCTTG
Transferrin	ATTCTCAAAGTGGCACAGGAACAC	GGGTTCTTTCCTTCGGTGTTATCC
Wnt1	TCTTCGGCAAGATCGTCAACCG	AAGGTTCATGAGGAAGCGTAGG

(Atlanta Biologicals), 2 mM glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 0.1 mM β-mercaptoethanol (Invitrogen), and 1,000 U of leukemia inhibitory factor (LIF) (Chemicon)/ml. Five days after plating, the inner cell mass was treated with trypsin and harvested, and replated on feeder cell layers in 24-well dishes. ES cell colonies were replated on feeder cell layers every 2 to 3 days.

Retroviral transduction studies were performed by using the vector pMSCV-Flag-JNK1-IRES-Puro (31). ES cells were plated on puromycin resistant RJ feeder layers (provided by Stephen N. Jones, University of Massachusetts Medical School) in ES cell growth medium (48 h) prior to infection. The medium was replaced at 24 h postinfection with fresh medium supplemented with 2 μg of puromycin/ml. Positive ES cell clones were screened for expression of the JNK1 polypeptide by immunoblot analysis.

Embryoid bodies (EBs) were cultured by plating single cells in suspension using DMEM supplemented with 10% fetal bovine serum (Invitrogen), 10% newborn calf serum (Invitrogen), 1% horse serum (Invitrogen), and 2 mM glutamine using uncoated petri dishes (8 days) and then 0.1% gelatin-coated dishes (7 days). The number of cells was measured by transferring 100 EBs to 1 ml of phosphate-buffered saline (PBS) in one well of a 24-well culture plate. These EBs were incubated with trypsin and dissociated to a single-cell suspension, and the number of cells was measured by using a hemacytometer.

Teratomas. ES cell-derived teratomas were prepared by subcutaneous injection of 4×10^6 ES cells into the flanks of C57BL/6J-*scid* mice. The mice were euthanized at 3 weeks postinjection. The teratomas were removed, washed in PBS, fixed (14 h) in 4% paraformaldehyde, and processed for histology.

Cardiomyocyte differentiation *in vitro*. ES cell-derived cardiomyocytes were prepared *in vitro* (61). The ES cells were cultured using dishes coated with 0.1% gelatin (3 days) in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 10% newborn calf serum (Invitrogen), 1% horse serum (Invitrogen), 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1,000 U of LIF/ml, and 0.15 μg of Noggin-Fc (R&D Systems)/ml. Single cells were cultured in the same medium without LIF and β-mercaptoethanol (1 day) and then in the same medium without LIF (Chemicon), β-mercaptoethanol, and Noggin-Fc (7 days) in suspension to form EBs using uncoated petri dishes. The EBs were transferred to gelatin-coated tissue dishes and cultured for 16 days.

Immunohistochemistry. Paraformaldehyde-fixed tissue was processed, embedded in paraffin, and 4-μm sections were stained with hematoxylin and eosin (H&E). Sections were also stained with antibodies to insulin (Dako), α-fetoprotein (Meridian Life Science), troponin I (Santa Cruz), keratin 5/8 (Lab Vision), desmin (Dako), Nestin (Chemicon), and the cytokeratin Endo-A (TROMA-1; Developmental Studies Hybridoma Bank, University of Iowa) using indirect immunoperoxidase detection (58). Alkaline phosphatase activity was measured using cells fixed with 4% paraformaldehyde at room temperature (1 h) using the Red Alkaline phosphatase substrate kit I (Vector). TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assays were performed by using an in situ cell death detection kit (Roche).

Immunofluorescence. Staining was performed using coverslips washed in PBS, incubated in 90% methanol containing 5% acetic acid at −20°C (5 min), washed with PBS, and then blocked by incubation with PBS supplemented with 1% skimmed milk for 1 h at room temperature and incubated with antibodies to SSEA-1 (Developmental Hybridoma Cell Bank, University of Iowa), Oct-4 (Chemicon), or FGF-4 (Santa Cruz, CA) in PBS containing 1% skimmed milk at 4°C (14 h). The cells were washed and then incubated with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 546 (Molecular Probes) at 37 °C (1 h). Washed slides were mounted and examined by using a Leica SP2 laser-scanning confocal fluorescence microscope.

Bromodeoxyuridine (BrdU) incorporation was examined by addition of 10 μM BrdU (Sigma) to the EB culture medium for 24 h. EB were fixed with 4% paraformaldehyde at 4°C (14 h) and embedded in OCT compound. Frozen sections (4 μm) were prepared, stained with a fluorescein isothiocyanate-conjugated antibody to BrdU (BD-Pharmingen), and examined by fluorescence microscopy (59).

Electron microscopy. Cells were fixed with 1.25% glutaraldehyde (30 min) at room temperature and with 2.5% glutaraldehyde in cacodylate buffer (14 h) at 4°C. The cells were then postfixed with 1% (wt/vol) osmium tetraoxide in PBS, dehydrated, and embedded in Lx 112/Araldite 502 epoxy resin. Ultrathin sections were mounted on copper support grids in serial order, contrasted with lead citrate and uranyl acetate, and examined on a Philips CM 10 transmission electron microscope (20).

Immunoblot analysis. Cell extracts were prepared by using Triton lysis buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of aprotinin and leupeptin/ml). Extracts (50 μg) were examined by immunoblot analysis. The blots were probed with antibodies to JNK (BD-Pharmingen), p38, ERK1, ERK2 (Santa Cruz), and α-tubulin (Sigma). Immunocomplexes were detected by enhanced chemiluminescence (NEN).

RNA analysis. Total RNA was prepared by using an RNeasy lipid tissue minikit (Qiagen) and was treated with DNase (Sigma). First-strand cDNA was synthesized by using an iScript cDNA synthesis kit (Bio-Rad). PCR analysis was performed using amplimers (Table 1).

Statistical analysis. Values are expressed as the mean ± the standard deviation. Comparisons among values for all groups were determined using a Student *t* test.

RESULTS

Isolation of JNK-deficient ES cells. We prepared E3.5 blastocysts from C57BL/6J mice and isolated ES cells in culture

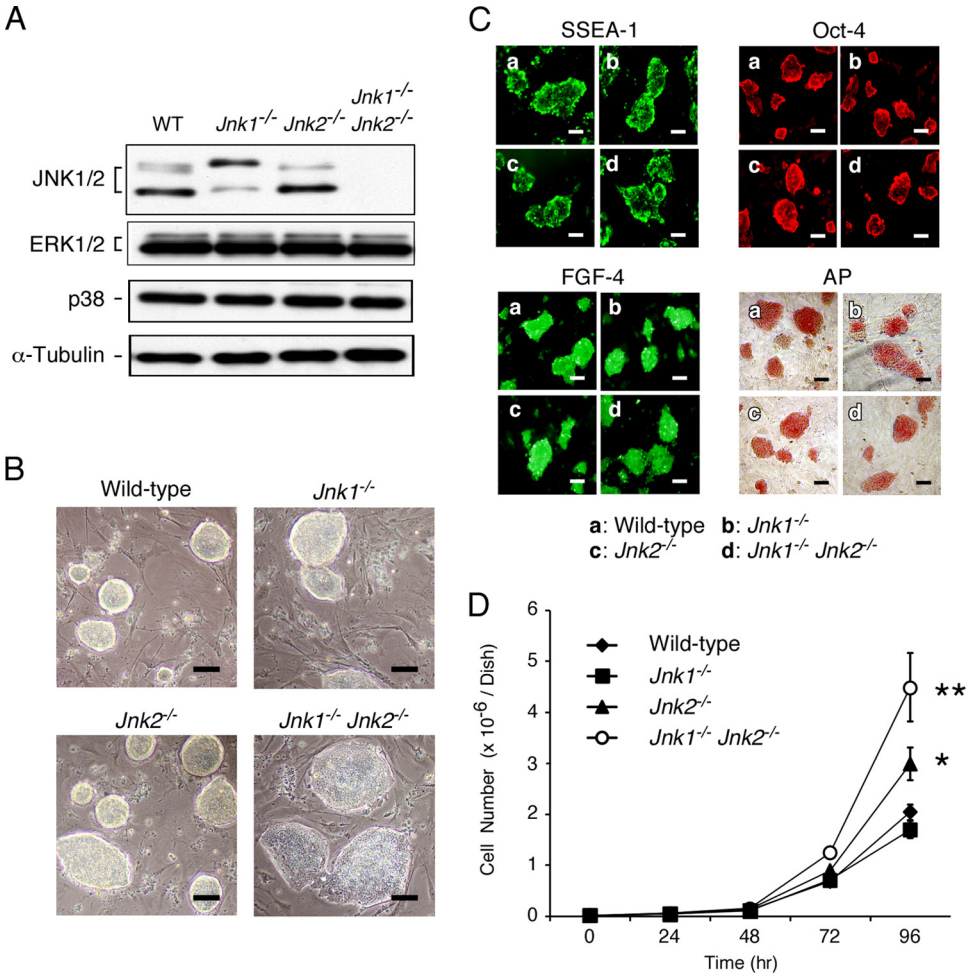


FIG. 1. Isolation of JNK-deficient ES cells. (A) Wild-type, *Jnk1*$^{-/-}$, *Jnk2*$^{-/-}$, and *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells were examined by immunoblot analysis with antibodies to JNK1/2, ERK1/2, p38 MAPK, and α-tubulin. (B) Wild-type and JNK-deficient ES cells grown on a layer of feeder cells (92 h) were examined by phase-contrast microscopy. Scale bar, 40 μm. (C) ES cells were stained for alkaline phosphatase activity and with antibodies to SSEA-1, Oct-4, and FGF-4. Scale bar, 40 μm. (D) Wild-type and JNK-deficient ES cells (10^4 cells) were plated in six-well gelatin-coated dishes. The number of cells on different days was measured. The data are shown as means ± the standard deviations (SD) ($n = 3$). Statistically significant differences are indicated (*, $P < 0.05$; **, $P < 0.01$).

using a feeder layer of inactivated MEFs. Studies using mice without or with targeted disruption of the *Jnk1* and *Jnk2* genes led to the isolation of wild-type ES cell clones and also JNK-deficient ES cell clones lacking expression of *Jnk1* and/or *Jnk2*. These clones were screened for the presence of X and Y chromosomes, and 18 male (XY) ES cell clones were selected for further study.

Representative ES cell clones were expanded in culture and examined by immunoblot analysis. JNK isoforms (46 and 55 kDa) were detected in lysates prepared from wild-type ES cells (Fig. 1A). Reduced expression of 46- and 55-kDa JNK isoforms was detected in lysates prepared from *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ ES cells, respectively. In contrast, no JNK was detected in lysates prepared from compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells (Fig. 1A). Control studies demonstrated that these ES cells did not express different levels of other MAP kinases (ERK1/2 and p38α).

The wild-type and JNK-deficient ES cells grow as colonies on feeder layers. No morphological differences between wild-type and JNK-deficient ES cell colonies were detected, although the compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells formed larger colonies than did the other ES cells (Fig. 1B). All of the ES cells we isolated expressed phenotypic markers that characterize an undifferentiated state (Fig. 1C), including alkaline phosphatase, SSEA-1, Oct-4, and FGF-4 (28). Together, these data indicate that JNK deficiency does not prevent the isolation of ES cells that express markers of the undifferentiated state.

JNK is not essential for ES cell proliferation. Compound JNK deficiency in MEFs causes severe growth retardation and early senescence (12, 54). We therefore examined the growth of wild-type and JNK-deficient ES cells grown as colonies. The proliferation of wild-type and *Jnk1*$^{-/-}$ ES cells was similar (Fig. 1D). In contrast, *Jnk2*$^{-/-}$ ES cells grew more rapidly than wild-type ES cells and compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells exhibited markedly increased proliferation compared to wild-type ES cells (Fig. 1D). The high proliferation rate of *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells suggests that the normal function of

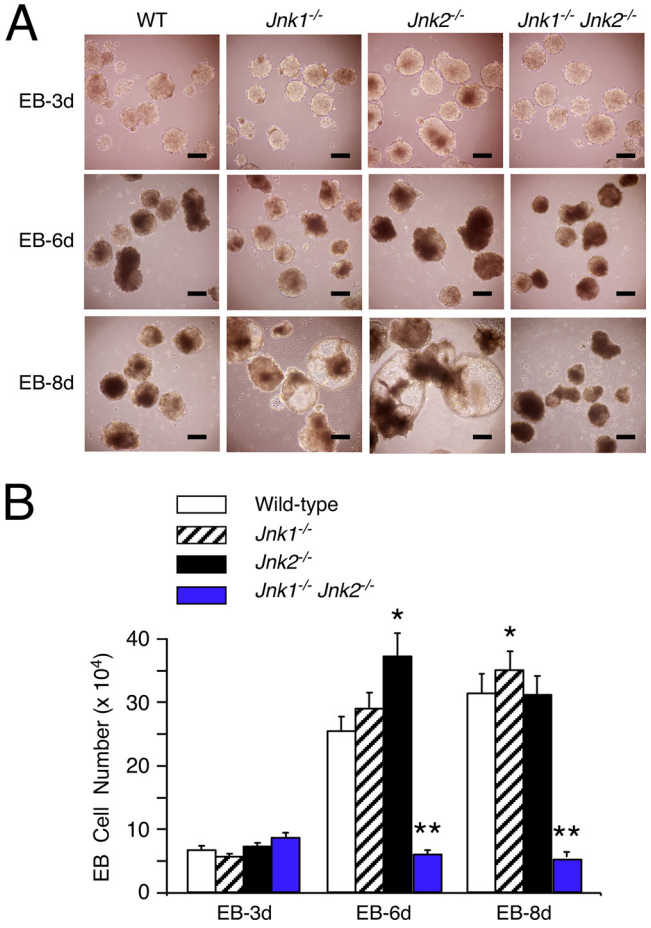


FIG. 2. Effect of JNK deficiency on the formation of EBs. (A) EBs at days 3, 6, and 8 were examined by phase-contrast microscopy. Scale bar, 250 μm. (B) The number of cells per EB was measured. The data are presented as the means ± the SD ($n = 100$). Statistically significant differences are indicated (*, $P < 0.05$; **, $P < 0.001$).

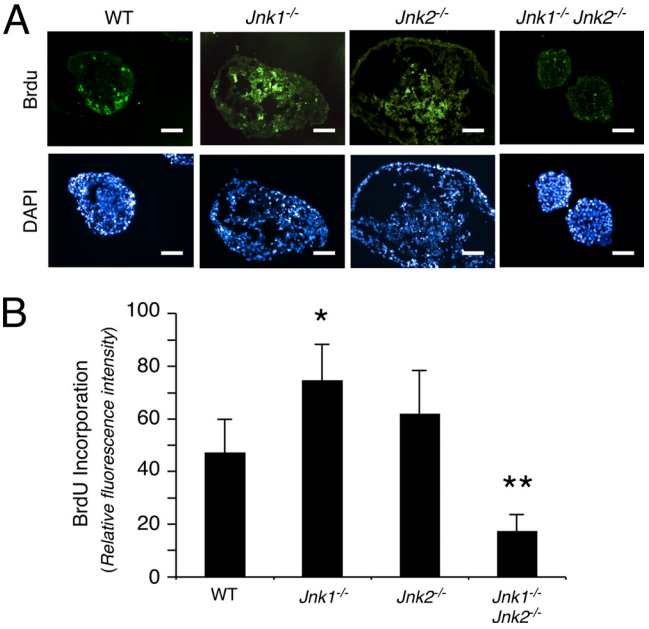


FIG. 3. Effect of JNK deficiency on proliferation. (A) Day 7 EBs were pulse-labeled with BrdU and fixed. Frozen sections were stained with DAPI (4′,6′-diamidino-2-phenylindole) and an antibody to BrdU and then examined by immunofluorescence analysis. Scale bar, 150 μm. (B) The mean fluorescence intensity of the BrdU immunofluorescence was measured by using ImageJ software and is presented as the means ± the SD ($n = 100$ EBs). Statistically significant differences are indicated (*, $P < 0.05$; **, $P < 0.01$).

JNK may be to limit ES cell proliferation and most likely accounts for the observation that these cells form large colonies on feeder cell layers (Fig. 1B). These data demonstrate that JNK is not required for ES cell proliferation. This observation contrasts with the finding that JNK deficiency causes rapid senescence in MEFs (12, 54).

JNK is required for the normal development of ES cell-derived EBs. To examine the properties of wild-type and JNK-deficient ES cells, we investigated the ability of these ES cells to form EBs. Wild-type ES cells, *Jnk1*$^{-/-}$ ES cells, and *Jnk2*$^{-/-}$ ES cells formed EBs that increased in size (Fig. 2A) and cell number (Fig. 2B) during culture *in vitro*. The *Jnk1*$^{-/-}$ EBs, and especially the *Jnk2*$^{-/-}$ EBs, formed larger luminal spaces than wild-type EBs (Fig. 2A). However, the proliferation of wild-type, *Jnk1*$^{-/-}$, or *Jnk2*$^{-/-}$ cells in the EBs was similar (Fig. 2B). This finding contrasts with the observation that undifferentiated *Jnk2*$^{-/-}$ ES cell colonies proliferated more rapidly than wild-type or *Jnk1*$^{-/-}$ ES cell colonies in culture (Fig. 1D).

The compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells also formed EBs (Fig. 2A). These structures lacked the large luminal spaces found in *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ EBs (Fig. 2A) and the number of cells within the compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ EBs did not increase during culture *in vitro* (Fig. 2B). Thus, while JNK is not required for ES cell proliferation (Fig. 1D), JNK is required for proliferation of cells that have initiated a differentiation program (Fig. 2B). This stage-specific role for JNK correlates with a change in the rapid cell cycles of ES cells to the slower cell cycles of differentiated cells that include fully formed gap (G_1/G_2) phases.

Previous studies have implicated JNK in both proliferation and death (14). The effect of compound JNK deficiency to prevent the accumulation of cells within EBs may therefore be caused by changes in either cellular proliferation or death. To test this hypothesis, we examined proliferation in EBs by investigating the incorporation of BrdU into DNA (Fig. 3) and cell death by TUNEL assays (Fig. 4). These studies demonstrated that the compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ EBs exhibited decreased DNA synthesis and also decreased apoptosis. The failure of compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ EBs to grow (Fig. 2B) is therefore accounted for by decreased proliferation rather than increased cell death.

Role of JNK in the expression of endodermal lineage genes *in vitro*. Development of the endoderm is one of the earliest steps in embryonic development and genes associated with endodermal differentiation (e.g., *Apoa2*, *Hnf1*, *Hnf3*, *Sox17*, and *Transferrin*) are expressed during the formation of ES cell-derived EBs. *Transferrin* and *Hnf3* are essential for differentiation of the visceral endoderm (7, 17) and show a similar pattern of expression in EBs formed by wild-type and JNK-deficient ES cells (Fig. 5A). Similarly, JNK deficiency did not cause major changes in the expression of *Apoa2*, a gene that is

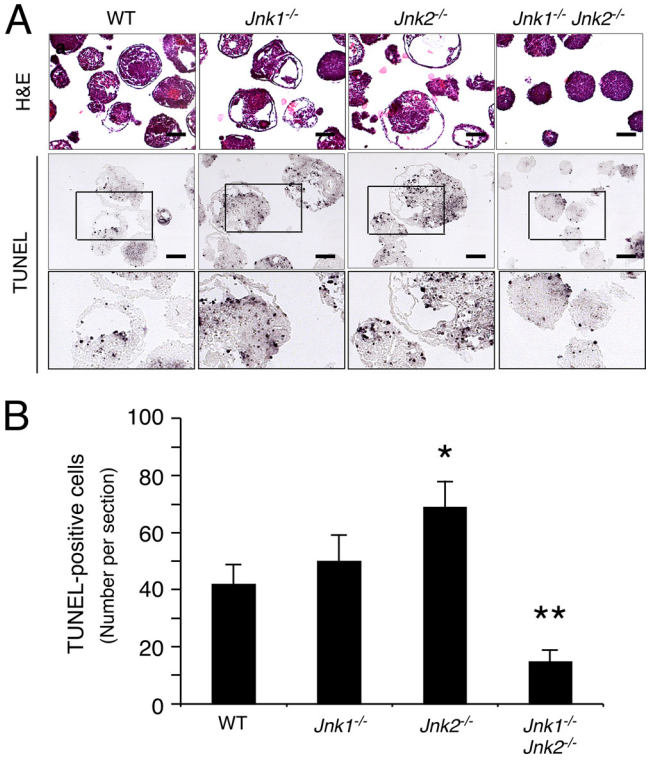


FIG. 4. Effect of JNK deficiency on apoptosis. (A) Day 7 EBs were fixed and embedded in paraffin. Sections were stained with H&E and examined by TUNEL assay. Scale bar, 250 μm. A higher-magnification image of a selected region (indicated with a box) of the TUNEL assay is also shown. (B) The number of TUNEL-positive cells in sections of EBs was measured and is presented as the mean ± the SD ($n = 100$ EBs). Statistically significant differences are indicated (*, $P < 0.05$; **, $P < 0.01$).

critically required for liver development (53). However, we found JNK-dependent differences in the expression of *Sox17* and *Hnf1*, genes that are required for differentiation of definitive endoderm (27) and visceral endoderm (5), respectively. Thus, *Sox17* expression was downregulated in late-stage EBs formed by JNK1/2-deficient ES cells compared to wild-type ES cells (Fig. 5A). In contrast, the expression of *Hnf1* was increased at the early stages of EB formation by JNK1/2-deficient ES cells compared to wild-type ES cells (Fig. 5A). Together, these data indicate a nonessential role of JNK for endodermal differentiation, but JNK may influence this developmental program.

Role of JNK in the expression of mesodermal lineage genes *in vitro*. Members of the transforming growth factor-β superfamily, including *Bmp4* and *Nodal*, are implicated in the induction of mesoderm and gastrulation (4). *Nodal* was expressed at similar levels in wild-type and JNK1/2-deficient EBs, but some variations in *Bmp4* expression were detected (Fig. 5B). These data suggest that the initial development of mesoderm may be JNK independent. Nevertheless, *Brachyury*, a gene that is critical for mesoderm development (22), was poorly expressed by EBs formed by compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells (Fig. 5B). This observation suggests that JNK may play an important role in the development of mesodermal lineages.

To further examine the JNK dependence of mesodermal lineage gene expression, we examined genes related to cardiomyogenesis, including *Flk1*, *Gata4*, *Nkx2.5*, *Mef2c*, β-*Mhc*, *Mlv2v*, and *Anf*. These genes play important roles in the specification of cardiac progenitor cells and cardiac development (6, 9, 19, 24, 29, 33, 35, 44, 45, 51). Expression of *Gata4* and *Mef2c* transcription factor mRNA was markedly reduced in JNK1/2-deficient EBs at early stages of differentiation compared to wild-type EBs (Fig. 5B). At late stages of differentiation, very low levels of *Flk1*, *Mef2c*, β-*Mhc*, *Mlv2v*, and *Anf* gene expression were detected in EBs formed by compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells (Fig. 5B). These data strongly support the conclusion that the JNK pathway is important for mesodermal differentiation, including the development of cardiac cells *in vitro*.

Role of JNK in the expression of ectodermal lineage genes *in vitro*. We examined whether JNK deficiency affected the development of ectoderm in ES cell-derived EBs. *Keratin17* is a marker for epidermal cells (41), and its expression was modestly decreased in EBs formed by compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells compared to wild-type ES cells at late stages of development (Fig. 5C). These data suggest that JNK is not critically required for ectodermal differentiation.

Compound deficiency of *Jnk1* and *Jnk2* in embryos causes early death associated with defects in neurogenesis (32). We therefore examined the expression of genes related to early neurogenesis in wild-type and JNK-deficient EBs. The expression of *Emx2*, a gene that is essential for forebrain development (37), was decreased in JNK1/2-deficient EBs compared to wild-type EBs (Fig. 2D). Expression of *Otx1*, a marker for early neuroectodermal development that is expressed at the 1-3 somite stage in the anterior neuroectoderm of mouse embryos (10), was increased in early stage JNK1/2-deficient EBs (Fig. 5C). It has been established that *Wnt1* and *Pax2* regulate mesencephalon and metencephalon development (38), and the expression of these genes was markedly downregulated in EBs formed by compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells compared to wild-type ES cells (Fig. 5C). Together, these findings support the conclusion that JNK contributes to ectoderm development and indicate that JNK-deficient ES cells exhibit abnormal neurogenesis *in vitro*.

Role of JNK in differentiation *in vivo*. *In vitro* analysis of ES cells indicates that JNK can influence differentiation (Fig. 5). Marker gene expression analysis demonstrated that JNK deficiency caused large defects in the development of mesoderm and more limited defects in ectodermal and endodermal development *in vitro* (Fig. 5). Methods that can be used to study differentiation *in vivo* include the analysis of mutant and mosaic mice. However, these approaches were not feasible for the analysis of JNK function because *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ mice die during early embryonic development (32) and mosaic mice prepared by injection of *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells into wild-type blastocysts exhibit low levels of chimerism (15). Therefore, to test the role of JNK in differentiation *in vivo*, we examined the formation of ES cell-derived teratomas, including tissues formed by all three germ cell layers (Fig. 6 and 7).

Histological analysis of sections prepared from teratomas demonstrated the presence of endodermal tissues (Fig. 6A). Stratified columnar ciliated epithelium originates from the endoderm (28) and a modest reduction in the amount of this

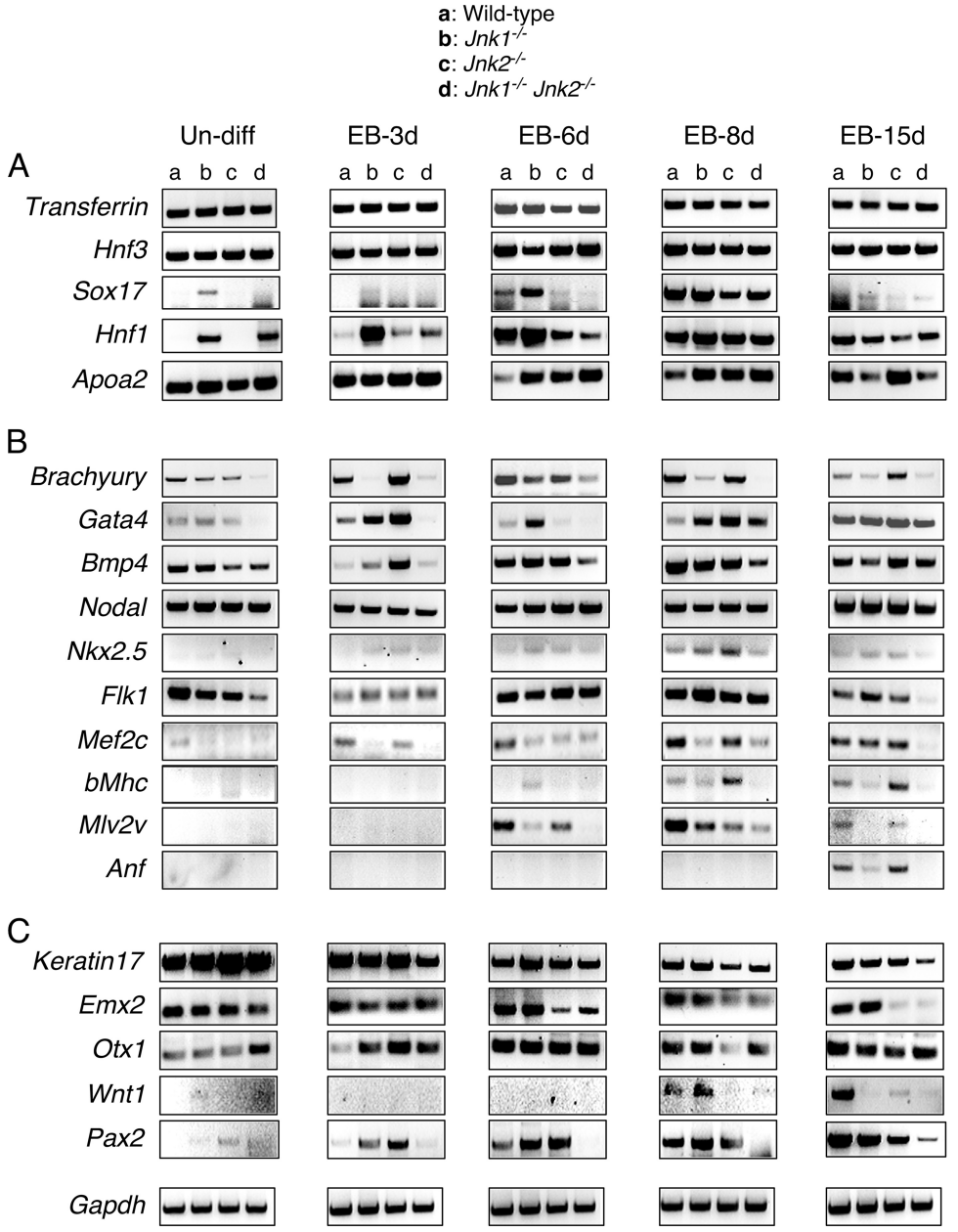
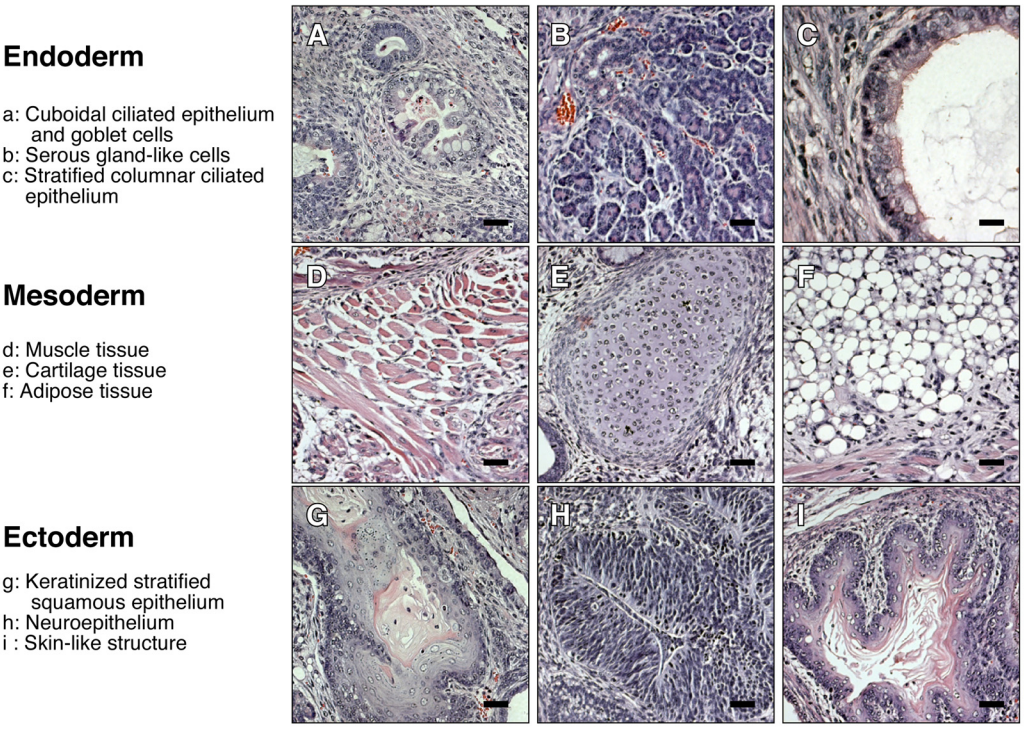


FIG. 5. Effect of JNK deficiency on differentiation-associated gene expression *in vitro*. Semiquantitative reverse transcription-PCR (RT-PCR) analysis was performed to detect the expression of mRNA associated with differentiation to endoderm (A), mesoderm (B), and ectoderm (C). Total RNA from undifferentiated ES cells (un-diff) or various stages of EB development and differentiation between days 3 and 15 (EB-3d to EB-15d) was examined. The amplimers used for this analysis are listed in Table 1. *Gapdh* gene expression was analyzed as an internal control. The results are representative of three independent experiments.

tissue was detected in JNK-deficient teratomas (Fig. 6B). However, JNK deficiency was found to cause no difference in tissues with serous gland-like cells or cuboidal ciliated epithelium (Fig. 6B). Immunohistochemical staining of tissue sections for the cytokeratin Endo-A (an endodermal marker) with the TROMA1 antibody (30) did not reveal differences between wild-type and JNK-deficient teratomas (data not shown). However, in contrast to wild-type teratomas, decreased numbers of hepatocytelike cells (that stain with an antibody to α-fetoprotein) and no insulin-positive pancreatic β-like cells were detected in compound mutant $Jnk1^{-/-}$ $Jnk2^{-/-}$ teratomas (Fig. 7A). These data indicate that JNK is not essential for endodermal development, but JNK may influence differentiation of some specific cell types derived from the endoderm.

Tissues with a mesodermal origin in wild-type and JNK-deficient teratomas, including muscle, cartilage, and adipose tissue, were examined. Major reductions in the amount of all three tissues were detected in JNK-deficient teratomas compared to wild-type teratomas (Fig. 6B). Immunohistochemical staining of tissue sections using antibodies to cardiac muscle

A



B

Tissue types		Wild-type	*Jnk1*$^{-/-}$	*Jnk2*$^{-/-}$	*Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$
Endoderm	Cuboidal ciliated epithelium and goblet cells	+++	++++	+++	+++
	Serous gland-like cells	+++	+++	++	+++
	Stratified columnar ciliated epithelium	+++	++	++	++
Mesoderm	Muscle tissue	+++	+++	++++	+
	Cartilage tissue	+++	+/-	++	-
	Adipose tissue	+++	+	++	+/-
Ectoderm	Keratinized stratified squamous epithelium	+++	+++	+++	+/-
	Neuroepithelium	+++	++	++	++
	Skin-like structure	+++	+++	++++	+/-

FIG. 6. Effect of JNK deficiency of ES differentiation *in vivo*. (A) Subcutaneously injected ES cells form teratomas in congenic mice. H&E-stained sections of teratomas formed by wild-type ES cells show the formation of endodermal (a to c), mesodermal (d to f), and ectodermal (g to i) tissues. Scale bar, 60 μm. (B) Comparison of teratomas derived from wild-type and JNK-deficient ES cells. Different tissues within sections of teratomas were identified and the area within each section was determined by using ImageJ software. The relative expression of each tissue within teratomas formed by ES cells with different genotypes is presented with a scale from "−" (lowest) to "++++" (highest). This scale is defined in Table 2.

troponin I and striated muscle desmin indicated the presence of both cardiomyocytelike and striated-muscle-like cells in wild-type teratomas, but no cardiac muscle tropinin I and significantly decreased striated muscle desmin were detected in compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ teratomas (Fig. 7B). These data indicate that mesoderm development is markedly impaired by JNK deficiency *in vivo*.

We examined tissues with an ectodermal origin in wild-type and JNK-deficient teratomas, including keratinized stratified squamous epithelium, neuroepithelium, and skinlike struc-

TABLE 2. Quantitation of teratoma cell type[a]

Tissue type	Mean % area					
	++++	+++	++	+	+/–	–
Cuboidal ciliated epithelium and goblet cells	>15	>10	>5	>1	>0.1	0
Serous glandlike cells	>0.4	>0.3	>0.2	>0.1	>0.01	0
Stratified columnar ciliated epithelium	>4	>3	>2	>1	>0.1	0
Muscle	>20	>15	>10	>5	>0.1	0
Cartilage	>0.5	>0.1	>0.05	>0.01	>0.005	0
Fat	>0.5	>0.1	>0.05	>0.01	>0.005	0
Keratinized stratified squamous epithelium	>0.5	>0.1	>0.05	>0.01	>0.005	0
Neuroepithelium	>30	>20	>10	>1	>0.1	0
Skinlike structures	>1	>0.5	>0.1	>0.05	>0.01	0

[a] Sections of teratomas stained with H&E were examined to identify differentiated cell types (Fig. 6). The teratoma area for each differentiated cell type was quantitated using ImageJ software ($n = 20$). The mean percent area for each cell type was calculated and is represented on a scale from highest ("++++") to lowest ("–") as discussed in the text.

tures. Studies of compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ teratomas demonstrated reduced amounts of all three ectodermal tissues compared to wild-type teratomas (Fig. 6B). Immunohistochemical staining of tissue sections using an antibody to keratin 5/8, a marker for epidermal skin cells, demonstrated markedly reduced expression in compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ teratomas compared to wild-type teratomas (Fig. 7C). A small decrease in Nestin expression (a marker of primitive neuroectoderm) was also detected in compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ teratomas compared to wild-type teratomas (Fig. 7C). Together, these data indicate that JNK can play an important role in ectoderm development *in vivo*.

JNK is not required for ES cell self-renewal. Studies of ES cells *in vitro* and *in vivo* demonstrate that JNK deficiency causes lineage-specific defects in differentiation (Fig. 5 to 7). These defects in differentiation may result from either a role of JNK in the differentiation program or because JNK-deficient ES cells have lost their differentiation potential due to a failure of self-renewal. To distinguish between these possibilities, we performed complementation analysis using recombinant JNK expressed in compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells. The rationale for these studies was that if the ES cells exhibited a failure of self-renewal, genetic complementation would not lead to restoration of differentiation potential. In contrast, if the JNK-deficient ES cells underwent self-renewal and JNK was required for differentiation, genetic complementation would restore differentiation potential.

We examined ES cells using a Noggin-induced differentiation protocol that leads to the formation of cardiomyocytes (61). Indeed, spontaneous beating of cell sheets was reproducibly detected within 6 days after attachment of wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ EBs in culture (Fig. 8A; see also Videos S1 to S3 in the supplemental material). Within 16 days after attachment, spontaneous beating of cells with a distinct muscle morphology was observed (Fig. 8A; see also Videos S5 to S7 in the supplemental material). Spontaneous beating is a property of both cardiomyocytes and smooth muscle cells. To confirm that these cultures contain cardiomyocytes (61), we examined the differentiated cells by transmission electron microscopy. Sarcomeric and Z-bands structures were detected consistent with the presence of cardiomyocytes (Fig. 8B).

No spontaneously beating cell sheets or muscle were detected in cultures of compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ cells (Fig. 8A; see also Videos S4 and S8 in the supplemental material). Indeed, gene expression analysis demonstrated that the mesoderm marker *Brachyury* and the cardiomyocyte markers *Anf*, *Flk1*, *Mef2c*, β-*Mhc*, and *Mlv2v* expressed in wild-type cultures were not expressed in cultures of compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ cells (Fig. 8C). These data confirm that JNK is essential for the formation of cardiomyocytes.

Complementation tests were performed using retroviral transduction of epitope-tagged JNK1α1 in compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells. Immunoblot analysis of cell lysates demonstrated that the 46- and 55-kDa JNK isoforms present in wild-type ES cells were absent in JNK-deficient ES cells (Fig. 8D). However, epitope-tagged JNK1α1 was detected in JNK-deficient ES cells transduced with the JNK1α1 retroviral vector (Fig. 8D). The expression of JNK1α1 in compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells restored the wild-type ES cell phenotype, including gene expression (*Anf*, *Brachyury*, *Flk1*, *Mef2c*, β-*Mhc*, and *Mlv2v*) and the development of beating cardiomyocytes in culture (Fig. 8E; see Video S9 in the supplemental material). Furthermore, analysis of teratomas formed by the complemented JNK-deficient ES cells demonstrated the restoration of cardiomyocyte development *in vivo* (Fig. 8F). Together, these data confirm that JNK is required for the development of cardiomyocytes and demonstrate that JNK is not required for ES cell self-renewal.

DISCUSSION

Studies of primary MEFs demonstrate that *Jnk1*$^{-/-}$ MEFs proliferate more slowly than wild-type MEFs and that *Jnk2*$^{-/-}$ MEFs proliferate more rapidly than wild-type MEFs (54). This observation has been interpreted to indicate that JNK1 and JNK2 may have opposite roles during proliferation (48). More recent studies, using a chemical genetic approach, demonstrate that these *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ phenotypes reflect adaptation and gain-of-function of the remaining JNK isoform (25). Thus, *Jnk2*$^{-/-}$ MEFs have increased JNK1 protein kinase activity compared to wild-type MEFs (25). These adaptive changes may account for the effects of JNK1 or JNK2 deficiency on ES cells. For example, *Jnk1*$^{-/-}$ ES cells formed colonies more slowly and *Jnk2*$^{-/-}$ ES cells formed colonies more rapidly than wild-type ES cells (Fig. 1D). Similarly, the increased luminal space within JNK1- or JNK2-deficient EBs may reflect adap-

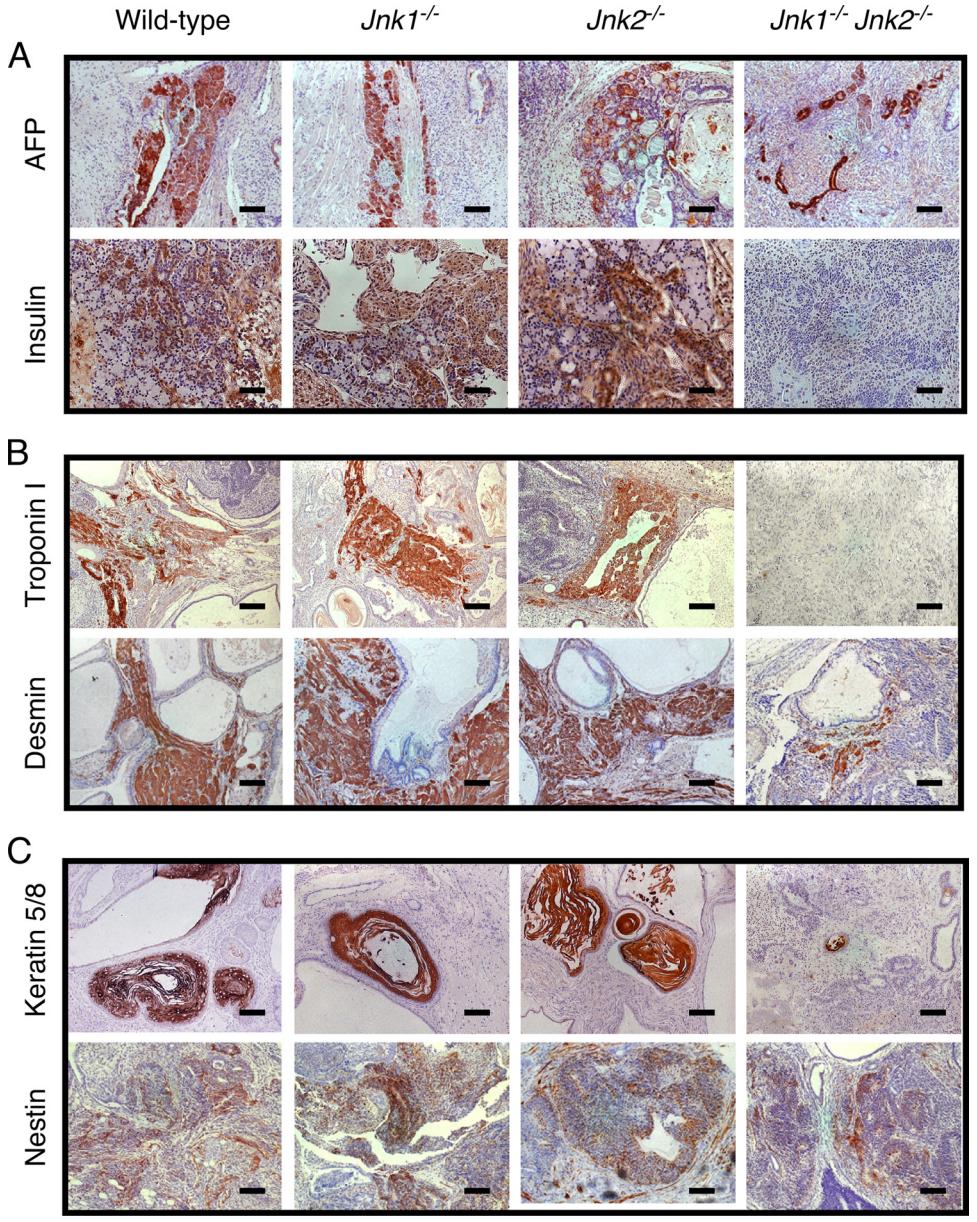


FIG. 7. Defective differentiation of JNK-deficient ES cells *in vivo*. Sections of teratomas formed by wild-type and JNK-deficient ES cells were examined by staining with H&E. (A) Hepatocytelike cells (upper panel) and β-like cells (lower panel) of endodermal origin were detected by staining with antibodies to α-fetoprotein (AFP) and insulin. Scale bars: upper, 250 μm; lower, 125 μm. (B) Cardiomyocytelike cells (upper panel) and striated-muscle-like cells (lower panel) of mesodermal origin were detected with antibodies to troponin I and desmin. Scale bars, 250 μm. (C) Keratinocytelike cells (upper panel) and primitive neuroepitheliumlike cells (lower panel) of ectodermal origin were detected with antibodies to keratin5/8 and nestin. Scale bars, 250 μm.

tation by the remaining JNK isoforms. Together, these considerations illustrate the need for studies of compound mutants that completely lack JNK expression.

It has been established that compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ MEFs proliferate slowly (54). Subsequent studies using conditional gene ablation and chemical genetic analysis demonstrated that loss of JNK function resulted in rapid p53-dependent senescence of MEFs (12). These data suggest that JNK plays a critical role in cell proliferation. However, our analysis of JNK-deficient ES cells indicates that the role of JNK may be more complex that previously anticipated. We show that compound mutant *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells proliferate more rapidly than wild-type ES cells when cultured as undifferentiated colonies on feeder layers of inactivated MEF (Fig. 1D). However, these JNK-deficient ES cells exhibit a marked proliferation defect when cultured as EBs (Fig. 2 and 3). This analysis demonstrates that there is no obligate role of JNK in the cell cycle, but JNK does influence cell cycle progression in a context-specific manner.

We speculate that these different roles of JNK in cell cycle regulation reflect the interaction of JNK with the p53 pathway. JNK deficiency does not cause growth inhibition in ES cells

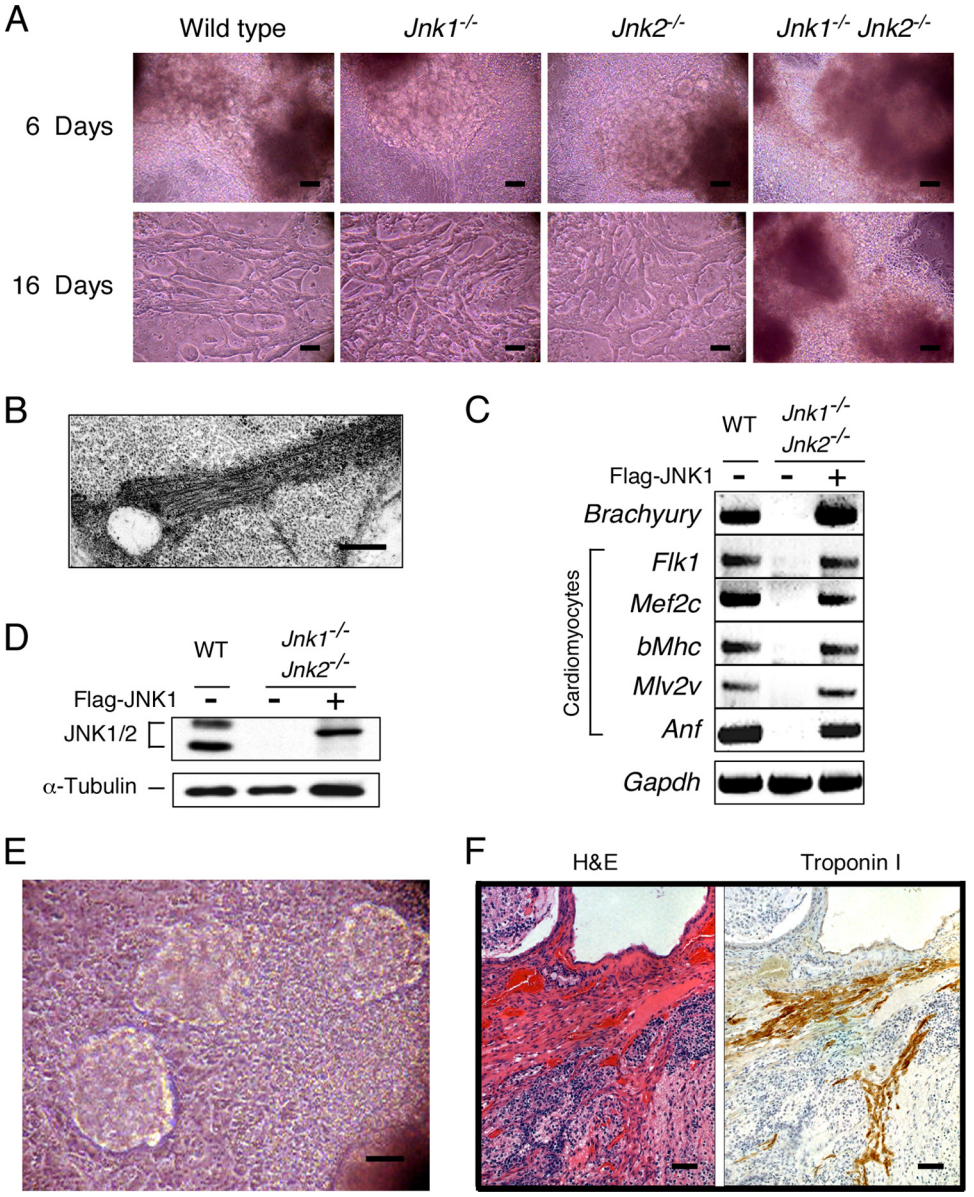


FIG. 8. JNK is not required for ES cell self-renewal. (A) ES cell-derived cardiomyocytes after 6 or 16 days of differentiation were examined by phase-contrast microscopy. Scale bar, 20 μm. The images represent single frames of movies (see Videos S1 to S8 in the supplemental material). (B) Cardiomyocytes derived from wild-type ES cells were examined by transmission electron microscopy. Sarcomeric and Z-band structures were detected. Scale bar, 1 μm. (C) Complementation analysis demonstrates that the expression of Flag-JNK1 in in *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells restored the expressed of mesodermal differentiation markers in EBs at 15 days. Total RNA was isolated and mRNA expression was examined by semiquantitative RT-PCR analysis. (D) Flag-JNK1 was expressed in *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells by retroviral transduction. The amount of JNK and α-tubulin in ES cell lysates was examined by immunoblot analysis. (E) Beating ES cell-derived cardiomyocytes in cultures of *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells that express Flag-JNK1 were detected by phase-contrast microscopy. Scale bar, 20 μm. The image represents a single frame of a movie (see Video S9 in the supplemental material). (F) Sections of teratomas derived from *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ ES cells that express Flag-JNK1 demonstrate the presence of cardiomyocytelike structures stained with H&E (left) and an antibody to troponin I (right). Scale bar, 250 μm.

that exhibit rapid cell cycles that lack fully formed gap (G_1/G_2) phases (57). Moreover, it is established that the p53 pathway fails to mediate growth arrest in ES cells (2, 23), in part, because of cytoplasmic retention of p53 (52). In contrast, ES cells grown in EBs initiate a differentiation program that changes the cell cycle to include fully formed gap (G_1/G_2) phases (57). Similarly, the cell cycle of MEFs includes fully formed gap phases (57). The effect of JNK deficiency to cause p53-dependent growth inhibition therefore correlates with the presence of gap (G_1/G_2) phases in the cell cycle.

JNK and developmental apoptosis. Cavity formation in EBs is a critical process that is required for differentiation and maturation (3, 40). The mechanism of cavity formation is mediated by programmed cell death of the inner ectodermal cells and survival of the outer ectodermal cells that form a columnar epithelium mediated by contact with the basement membrane

(11). This mechanism of dual signaling to selectively target ectodermal cells for survival and death is an essential step in development. Previous studies have established that compound mutant embryos lacking *Jnk1* and *Jnk2* exhibited severe dysregulation of cell survival and apoptosis (32). It was therefore possible that JNK may contribute to cavitation. Indeed, $Jnk1^{-/-}$ $Jnk2^{-/-}$ ES cells formed EBs without cavitation (Fig. 2). This loss of cavitation was associated with reduced apoptosis and also with reduced proliferation (Fig. 3 and 4). These data demonstrate that JNK is required for a very early morphogenetic process that involves developmentally regulated apoptosis. It is likely that the loss of cavitation has profound consequences for differentiation. This is consistent with the finding that $Jnk1^{-/-}$ $Jnk2^{-/-}$ ES cells exhibit a reduced capacity to differentiate (Fig. 5 to 8).

Stem cell self-renewal and differentiation. The observation that $Jnk1^{-/-}$ $Jnk2^{-/-}$ ES cells exhibit a profound defect in differentiation (Fig. 5 to 8) could reflect either a requirement of JNK for the maintenance of stem cell totipotency or a requirement of JNK for differentiation. We used genetic complementation assays to distinguish between these possibilities. We found that the ectopic expression of JNK in $Jnk1^{-/-}$ $Jnk2^{-/-}$ ES cells was fully capable of restoring the differentiation of these cells to a mesodermal lineage (Fig. 8). This finding demonstrates that the self-renewal potential of ES cells does not require JNK. However, JNK is required for specific differentiation programs. This conclusion is consistent with the finding that radiation chimeras prepared by transplantation of $Jnk1^{-/-}$ $Jnk2^{-/-}$ bone marrow into lethally irradiated recipient mice results in the reconstitution of these animals by $Jnk1^{-/-}$ $Jnk2^{-/-}$ hematopoietic stem cells (13).

Conclusions. We report that JNK is not required for stem cell self-renewal. However, JNK does play a major role in the suppression of senescence, proliferation, apoptosis, and differentiation of ES cells that have committed to a specific cell lineage.

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