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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 deficiency of either the Jnk1 or Jnk2 genes (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 pre-designed 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) suggest 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/Slc) 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 embryonic fibroblasts (MEFs) inactivated with mitomycin C (Sigma) in Dulbecco modified Eagle medium (DMEM; Invitrogen), 15% fetal bovine serum

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† Supplemental material for this article may be found at http://mcb.asm.org/.

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TABLE 1. Amplimers used in this study

<table>
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<th>Gene</th>
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We prepared E3.5 blastocysts from C57BL/6J mice and isolated ES cells in culture.

Cardiomyocyte differentiation in vitro. ES cell-derived cardiomyocytes were prepared in vitro (61). The ES cells were cultured in NIH-3T3 feeder 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-RES-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 petri 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.

Embryonic 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 petri 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.

Immunohistochemistry. Parafformaldehyde-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 1 (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 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.

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 room temperature. The cells were then postfixed with 1% (wt/vol) osmium tetroxide in PBS, dehydrated, and embedded in L12/Araldite 502 epoxy resin. Ultrathin sections were cut on a Leica EM 2002 ultramicrotome, 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-Phar- mingen), 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.
using a feeder layer of inactivated MEFs. Studies using mice without or with targeted disruption of the \textit{Jnk1} and \textit{Jnk2} genes led to the isolation of wild-type ES cell clones and also JNK-deficient ES cell clones lacking expression of \textit{Jnk1} and/or \textit{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 \textit{Jnk1}\textsuperscript{-/-} and \textit{Jnk2}\textsuperscript{-/-} ES cells, respectively. In contrast, no JNK was detected in lysates prepared from compound mutant \textit{Jnk1}\textsuperscript{-/-}\textit{Jnk2}\textsuperscript{-/-} 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 \textit{Jnk1}\textsuperscript{-/-}\textit{Jnk2}\textsuperscript{-/-} 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.

\textbf{FIG. 1.} Isolation of JNK-deficient ES cells. (A) Wild-type, \textit{Jnk1}\textsuperscript{-/-}, \textit{Jnk2}\textsuperscript{-/-}, and \textit{Jnk1}\textsuperscript{-/-} \textit{Jnk2}\textsuperscript{-/-} 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 (10⁴ 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).

\textbf{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 \textit{Jnk1}\textsuperscript{-/-} ES cells was similar (Fig. 1D). In contrast, \textit{Jnk2}\textsuperscript{-/-} ES cells grew more rapidly than wild-type ES cells and compound mutant \textit{Jnk1}\textsuperscript{-/-} \textit{Jnk2}\textsuperscript{-/-} ES cells exhibited markedly increased proliferation compared to wild-type ES cells (Fig. 1D). The high proliferation rate of \textit{Jnk1}\textsuperscript{-/-} \textit{Jnk2}\textsuperscript{-/-} ES cells suggests that the normal function of
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 (G1/G2) 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
cells (Fig. 5A). In contrast, the expression of Brachyury, a gene that is critical for mesoderm development (22), was poorly expressed by EBs formed by compound mutant Jnk1<sup>−/−</sup> Jnk2<sup>−/−</sup> ES cells compared to wild-type ES cells (Fig. 5A). 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<sup>−/−</sup> Jnk2<sup>−/−</sup> 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<sup>−/−</sup> Jnk2<sup>−/−</sup> 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<sup>−/−</sup> Jnk2<sup>−/−</sup> 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<sup>−/−</sup> Jnk2<sup>−/−</sup> mice die during early embryonic development (32) and mosaic mice prepared by injection of Jnk1<sup>−/−</sup> Jnk2<sup>−/−</sup> 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

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<sup>−/−</sup> Jnk2<sup>−/−</sup> 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.
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

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.
troponin I and striated muscle desmin indicated the presence of both cardiomyocyte-like and striated-muscle-like cells in wild-type teratomas, but no cardiac muscle troponin 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 \textit{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-
tured. 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 Mhv2v 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 Mhv2v) 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-

### TABLE 2. Quantitation of teratoma cell type

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>+++++</th>
<th>++++</th>
<th>+++</th>
<th>++</th>
<th>+</th>
<th>−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuboidal ciliated epithelium and goblet cells</td>
<td>&gt;15</td>
<td>&gt;10</td>
<td>&gt;5</td>
<td>&gt;1</td>
<td>&gt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>Serous glandlike cells</td>
<td>&gt;0.4</td>
<td>&gt;0.3</td>
<td>&gt;0.2</td>
<td>&gt;0.1</td>
<td>&gt;0.01</td>
<td>0</td>
</tr>
<tr>
<td>Stratified columnar ciliated epithelium</td>
<td>&gt;4</td>
<td>&gt;3</td>
<td>&gt;2</td>
<td>&gt;1</td>
<td>&gt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>Muscle</td>
<td>&gt;20</td>
<td>&gt;15</td>
<td>&gt;10</td>
<td>&gt;5</td>
<td>&gt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>Cartilage</td>
<td>&gt;0.5</td>
<td>&gt;0.1</td>
<td>&gt;0.05</td>
<td>&gt;0.01</td>
<td>&gt;0.005</td>
<td>0</td>
</tr>
<tr>
<td>Fat</td>
<td>&gt;0.5</td>
<td>&gt;0.1</td>
<td>&gt;0.05</td>
<td>&gt;0.01</td>
<td>&gt;0.005</td>
<td>0</td>
</tr>
<tr>
<td>Keratinized stratified squamous epithelium</td>
<td>&gt;0.5</td>
<td>&gt;0.1</td>
<td>&gt;0.05</td>
<td>&gt;0.01</td>
<td>&gt;0.005</td>
<td>0</td>
</tr>
<tr>
<td>Neuroepithelium</td>
<td>&gt;30</td>
<td>&gt;20</td>
<td>&gt;10</td>
<td>&gt;1</td>
<td>&gt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>Skinlike structures</td>
<td>&gt;1</td>
<td>&gt;0.5</td>
<td>&gt;0.1</td>
<td>&gt;0.05</td>
<td>&gt;0.01</td>
<td>0</td>
</tr>
</tbody>
</table>

* 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.
tation by the remaining JNK isoforms. Together, these considera-
tions 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. 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) Hepatocyctelike 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) Cardiomyocyctelike 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) Keratinocyctelike cells (upper panel) and primitive neuroepithelium-like cells (lower panel) of ectodermal origin were detected with antibodies to keratin5/8 and nestin. Scale bars, 250 μm.
that exhibit rapid cell cycles that lack fully formed gap (G1/G2) 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 (G1/G2) 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 (G1/G2) 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.
 ROLE OF JNK IN STEM CELLS

(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 caviation. Indeed, Jnk1−/− Jnk2−/− ES cells formed EBs without caviation (Fig. 2). This loss of caviation 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 caviation 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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