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Short Article

Caspase Activity Mediates the Differentiation of Embryonic Stem Cells

Jun Fujita,[1,7] Ana M. Crane,[1,7] Marlon K. Souza,[1,7] Marion Dejosez,[1] Michael Kyba,[4] Richard A. Flavell,[5] James A. Thomson,[6] and Thomas P. Zwaka[1,2,3,*]
[1]Center for Cell and Gene Therapy
[2]Department of Molecular and Cellular Biology
[3]Department of Molecular Human Genetics
Baylor College of Medicine, Houston, TX 77030, USA
[4]Department of Developmental Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
[5]Department of Immunobiology, Yale University School of Medicine and Howard Hughes Medical Institute, New Haven, CT 06520, USA
[6]The Wisconsin National Primate Research Center, University of Wisconsin, Madison, Madison, WI 53715, USA
[7]These authors contributed equally to this work.
*Correspondence: tpzwaka@bcm.edu
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SUMMARY

Embryonic stem cells (ESCs) are capable of indefinite self-renewal while retaining the ability to differentiate to any of the three germ layers that give rise to all somatic cell types. An emerging view is that a core set of transcription factors, including Oct4, Sox2, and Nanog, form a robust autoregulatory circuit that maintains ESCs in a self-renewing state. To accommodate the capacity of such cells to undergo germ layer-specific differentiation, we predicted a posttranslational mechanism that could negatively regulate these core self-renewal factors. Here we report caspase-induced cleavage of Nanog in differentiating ESCs. Stem cells lacking the *Casp3* gene showed marked defects in differentiation, while forced expression of a caspase cleavage-resistant Nanog mutant in ESCs strongly promoted self-renewal. These results link a major component of the programmed cell-death pathway to the regulation of ESC development.

INTRODUCTION

Embryonic stem cell (ESC) research holds remarkable promise, yet the mechanisms by which these cells transition from pluripotency to differentiation have been elusive. It now appears that a small core set of transcription factors work together to maintain the pluripotent state of ESCs (Bernstein et al., 2006; Boyer et al., 2005, 2006; Lee et al., 2006). These transcriptional regulators, including Oct4, Sox2, and Nanog, stimulate the expression of genes controlling self-renewal while repressing genes that drive differentiation. An emerging concept is that Nanog and other core transcription factors form a tight autoregulatory circuit that enables ESCs to remain stable in culture and ensures extreme autonomy in proliferative decisions (Boyer et al., 2005; Chickarmane et al., 2006). Thus, ESCs depend only marginally on mitogenic stimuli typically required for somatic cells to proliferate but stimulate their own growth through endogenous factors. This autonomy is best shown by the unique ability of ESCs, injected into virtually any anatomical site in adult animals, to form rapidly growing tumors called teratocarcinomas (Damjanov and Solter, 1974). How, then, do ESCs retain the capacity for rapid differentiation? The most plausible mechanism, in our view, would modify one or more core transcription factors posttranslationally, allowing the ESCs to rapidly escape the constraints of their self-renewal machinery.

Attractive candidates for the role of posttranslational modifier of ESC function are the site-specific proteases of the programmed cell death system (Earnshaw et al., 1999; Thornberry and Lazebnik, 1998). The cysteine protease Caspase-3 is especially notable because it not only cleaves vital proteins, but also activates other caspases, such as Caspase-9, that have their own targets. These proteases are very specific for particular amino acid sequences, are highly regulated in their activities, and, in some contexts, appear to influence the decision of cells to differentiate (Arama et al., 2003; De Botton et al., 2002; De Maria et al., 1999a, 1999b; Ishizaki et al., 1998), implying functions other than the execution of cell-death programs. Here we report that caspases play a critical role in ESC differentiation by negatively regulating the self-renewal machinery of these stem cells.

RESULTS

Caspase Activity Increases after Induction of ESC Differentiation

We first tested differentiating mouse ESC cultures for the presence of caspase activity. As shown in Figure 1A and Figure S1 available online, such activity began to increase very shortly after the ESCs were stimulated with retinoic acid (RA) or plated in differentiation medium. To exclude an effect from increased apoptosis, we assayed the cultures for the percentage of cells undergoing apoptosis, demonstrating essentially no increases in this end point over 72 hr poststimulation with RA (Figure 1B). To substantiate that the caspase activity peaks were associated with cell differentiation and not programmed cell death, we generated a caspase activity reporter cell line (Casp^{sensor}; Figure S2A) in which enhanced yellow fluorescent protein (EYFP) could

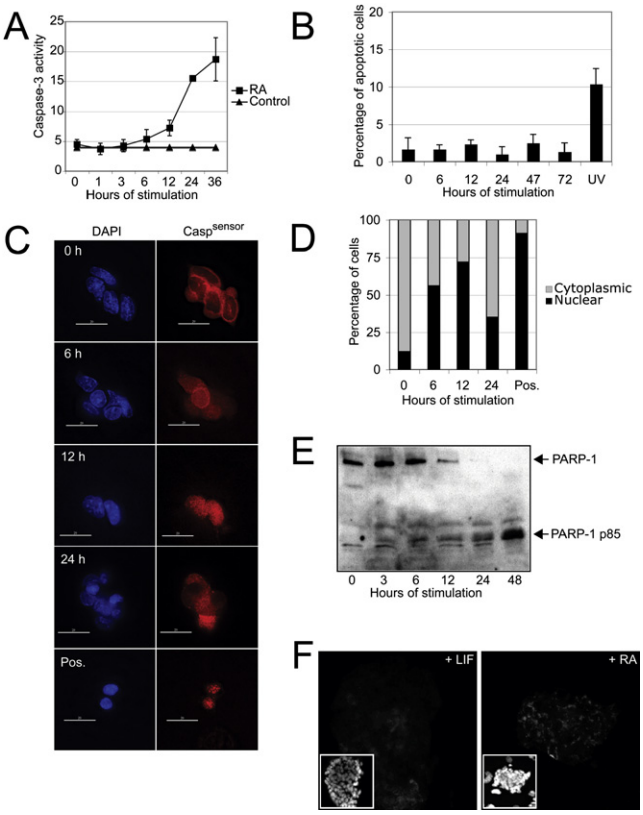

Figure 1. Increased Caspase Activity in Mouse ESCs upon Induction of Differentiation

(A) The R1 ESC line was stimulated with RA (1 μM) for the indicated times, and Caspase activity was measured in an in vitro Caspase activity assay. The data are means ± SD of triplicate experiments.

(B) The same ESC line was again exposed to RA for various times and the mean (± SD) percentage of cells undergoing programmed cell death was determined by counting apoptotic bodies. UV, ultraviolet light.

(C) ESCs expressing the Caspase sensor (Casp^{sensor}) were stimulated with RA (1 μM), fixed at the four indicated time points, and stained with an antibody against a reporter protein (enhanced yellow fluorescent protein, EYFP). Mainly cytoplasmic staining indicates low Caspase activity, while mainly nuclear staining typically indicates increased Caspase activity. Immunofluorescence images (40×) were taken from representative fields. Although a shift of the signal from the cytoplasm to the nucleus is apparent at 12 hr, the cells did not show any signs of apoptosis. Cells treated with stausporine at 6 hr served as positive controls. Scale bar, 10 μm.

(D) Similar experimental setting as in (C), except that the mean percentage of cells with mainly cytoplasmic or nuclear staining was determined.

(E) Western blot analysis of nuclear lysates isolated from mouse ESCs after stimulation with RA for the indicated times. The blotted membrane was probed with an antibody against PARP-1. The uncleaved form of PARP-1 is apparent at the earlier poststimulation times, with the cleaved form (85 kDa fragment) appearing after 24 hr, indicating PARP-1 cleavage.

(F) Immunostaining of a mouse ESC colony stimulated with RA or incubated with leukemia inhibitory factor (LIF) for 2 days. The antibody used specifically recognizes the 85 kDa form of PARP-1. Magnification, 40×.

be seen in the cytoplasm as long as caspase activity was low or absent but appeared in the nucleus when caspases were active (Figure 1C). In their undifferentiated state, the ESCs showed mainly cytoplasmic EYFP staining, but upon induction of differentiation, the EYFP signal shifted to the nucleus in most or an increased percentage of the ESCs (Figures 1C–1D), indicating the presence of caspase activity. Importantly, none of the caspase-positive cells appeared to be undergoing programmed cell death, as they lacked the classical features of nuclear condensation, nuclear fragmentation, and membrane blebbing (data not shown). Western blot analysis revealed marked differences in the sizes of the EYFPs, indicating that the reporter protein had indeed been cleaved by caspases (Figure S2B). We attribute the slight discrepancy in the kinetics of caspase activity shown in Figures 1A and 1D to the different cell densities required for the respective assays. Finally, we asked if PARP-1, a recognized caspase target during the execution of apoptosis (Lazebnik et al., 1994), might also be cleaved after induction of differentiation. Western blot analysis and immunofluorescence microscopy with PARP-1 antibodies revealed what appeared to be cleaved PARP-1 (the p85 fragment) at 48 hr poststimulation of ESCs (Figures 1E and 1F), suggesting that, as in cells undergoing apoptosis, PARP-1 was also cleaved by caspases during differentiation. Thus, differentiating ESCs show increased caspase activity that is not associated with programmed cell death.

Because many different caspases could have accounted for the increases in caspase activity seen in our differentiating ESC cultures, we performed a western blot analysis for active caspases in protein lysates from these cultures. Using antibodies against five major caspases, we identified active Caspase-3 in the lysates (Figure 2A) but failed to detect other effector cysteine proteases (data not shown). To demonstrate the functional relevance of Caspase-3 to ESC differentiation, we first exposed the cells to the caspase-blocking peptide VAD and induced differentiation with RA. This treatment clearly inhibited ESC differentiation, although the block was not complete (Figures 2B and 2D, left). Thus, while important for differentiation, Caspase-3 activity may not have been the sole factor contributing to this process. Alternatively, the VAD peptide may not have fully inactivated the protease.

Caspase-3 Knockout ESCs Possess a Differentiation Defect

To substantiate a requirement for Caspase-3 activity in ESC differentiation, we generated both homozygous and heterozygous lines of *Casp3* knockout ESCs (Figure S3A). Deletion of the *Casp3* locus lacked any discernible effect in undifferentiated ESCs; however, when exposed to RA, the *Casp3*$^{-/-}$ ESCs showed an obvious delay in differentiation compared with *Casp3*$^{+/-}$ cells (Figures 2C and 2D, right). Similar results were obtained when the ESCs were induced to differentiate as embryoid bodies (Figure S3B). As in the caspase blocking experiment, more than 25% of the *Casp3*$^{-/-}$ ESC colonies showed appreciable signs of differentiation after 5 days, while a substantial proportion of the colonies remained either completely undifferentiated (42%) or only partially differentiated (31%). Quantification of *Oct4* expression in *Casp3*$^{-/-}$ ESCs by PCR (Figure S3C and S3D) showed a reduction in this transcription factor with time after differentiation, reinforcing the idea that a differentiation delay is associated with the absence of this protease in ESCs. We also injected *Casp3*$^{+/-}$ and *Casp3*$^{-/-}$ ESCs into immunocompromised mice, observing tumor formation at the injection sites 10–14 days later in both experimental groups. Histological examination of the tumors revealed differentiated cells from all germ layers in mice injected with the *Casp3*$^{+/-}$ ESCs, in contrast

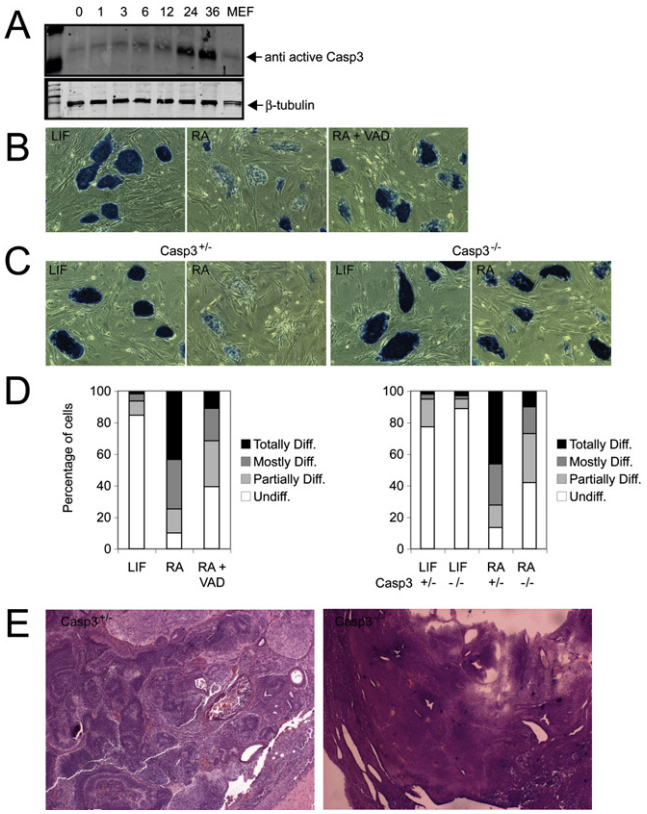

Figure 2. Caspase-3 Is Increased during Differentiation and Is Essential for Proper Differentiation of ESCs

(A) Western blot analysis of protein lysates from ESCs stimulated to differentiate with RA for the indicated times. The western blot membranes were probed with an antibody that specifically recognizes only active Caspase-3. MEF, mouse embryonic fibroblasts.

(B) Alkaline phosphatase staining of ESC colonies stimulated with RA (1 μM/ml) or coincubated with RA and the pan-Caspase blocking peptide VAD.fmk (100 μM) after 3 days. Cells incubated with leukemia inhibitory factor (LIF) served as controls. The majority of colonies lost staining due to differentiation, whereas coincubation with VAD prevented differentiation. Magnification, 10×.

(C) *Casp3* heterozygous (+/−) and knockout (−/−) ESCs were stimulated with RA (1 μM) for 3 days and stained for alkaline phosphatase. *Casp3*$^{+/-}$ ESCs differentiated, whereas the majority of *Casp3*$^{-/-}$ ESCs did not (10×). Controls were cells incubated without LIF.

(D) Left: quantification of colonies shown in (B) as described in the Experimental Procedures. Mean values are shown. Right: quantification of colonies shown in (C). Mean values are shown.

(E) *Casp3*$^{+/-}$ and *Casp3*$^{-/-}$ ESCs were injected into immunocompromised nude mice, and the resultant tumors were analyzed histologically. Typical aspects of *Casp3*$^{+/-}$ and of *Casp3*$^{-/-}$ tumors included derivatives from all three germ layers within a teratoma or immature and undifferentiated cells, respectively.

to the mainly undifferentiated or immature cells in *Casp3*$^{-/-}$ ESCs (Figure 2E; Figure S4). Taken together, the caspase-blocking and *Casp3* knockout data support direct involvement of Caspase-3 in ESC differentiation.

If Caspase-3 indeed promotes the differentiation of ESCs, it should be possible to demonstrate this effect by modulating levels of the active protease. We therefore made targeted insertions of cDNAs encoding a constitutively active (Casp3rev) or mutated (mCasp3rev) form of Caspase-3 upstream of the *HPRT* locus of A2lox ESCs; these cDNAs were under the control of a tetracycline-inducible promoter (Figure S5A). Doxycycline induction of higher levels of inducible Caspase-3 in ESCs stimulated differentiation, coinciding with a reduction of Nanog and Oct4 levels, an increase in the expression of miscellaneous differentiation factors, and an obvious change in cell morphology associated with differentiation (Figures 3A–3C). Whether such stimulation favors differentiation to a particular cell type (e.g., endoderm) or simply releases ESCs from the self-renewal machinery to be stimulated by other extrinsic or intrinsic signals required for cell-fate commitment is unclear.

Nanog Is Cleaved upon Induction of Differentiation

To identify the differentiation-specific molecular targets of Caspase-3 in mouse ESCs, we developed an in vitro Caspase-3 cleavage assay and used it to determine the cleavage of Sox2, Oct4, and Nanog transcription factors, all of which maintain ESCs in a self-renewing state. Caspase-3 cleaved human (h) Nanog in vitro (Figure 4A), while Sox2 and Oct4 remained uncleaved (data not shown). Examination of the hNanog amino acid sequence revealed conserved residues at position 69 and at position 70 between the N-terminal transcriptional transactivator (Pan and Pei, 2003) and the homeodomain (Figure 4A) that likely serve as the Caspase-3 cleavage site. Indeed, when a single amino acid in this putative recognition sequence was mutated (yielding D69E hNanog), Caspase-3 was no longer able to cleave the protein in vitro (Figure 4A). We also noted that mouse (m) Nanog has a single amino acid substitution (D > G) at position 64 that is three bases upstream of the Caspase-3 cleavage site, a modification that could decrease the ability of Caspase-3 to cleave mNanog. An in vitro Caspase-3 substrate affinity assay revealed that the cleavage site in mNanog (GSPD) was still targeted by Caspase-3 (Figure S6B). However, after performing the same in vitro cleavage assays as used with hNanog, we observed very little or no cleavage of recombinant mNanog by Caspase-3 (Figure S6A). To determine whether both hNanog and mNanog and their corresponding mutants are cleaved in differentiating ESCs in vivo, we studied all four constructs in ESCs treated with RA (Figure 4B). Western blot analysis revealed cleavage of both wild-type hNanog and wild-type mNanog, but not their mutant forms harboring modified caspase cleavage sites (aa 67 to 68 in mNanog and aa 69 to 70 in hNanog) (Figure 4B). In addition, western blot analysis with antibodies against endogenous Nanog detected a smaller band at 24 and 36 hr postinduction that corresponded to the cleaved form of Nanog (Figure 4C). Thus, given the apparently reduced activity of Caspase-3 toward mNanog in vitro compared with its effective cleavage of this transcription factor in vivo, we hypothesized that another, Caspase-3-dependent protease may function with Caspase-3 to modify the regulatory activity of mNanog. By screening a panel of caspases for their ability to cleave mNanog in vitro (Figure 4D, left), we identified Caspase-9 as the most likely candidate for this role (Figure 4D; Figure S6C). Indeed, others have shown that the activity of Caspase-9 can be drastically enhanced either by active Caspase-3 (Zou et al., 2003) or by Procaspase-3 (Yin et al., 2006), supporting an important contribution of Caspase-9 to Nanog cleavage in ESCs.

If Nanog is indeed one of the principal targets of Caspases in ESCs, its cleavage should have profound effects on whether the

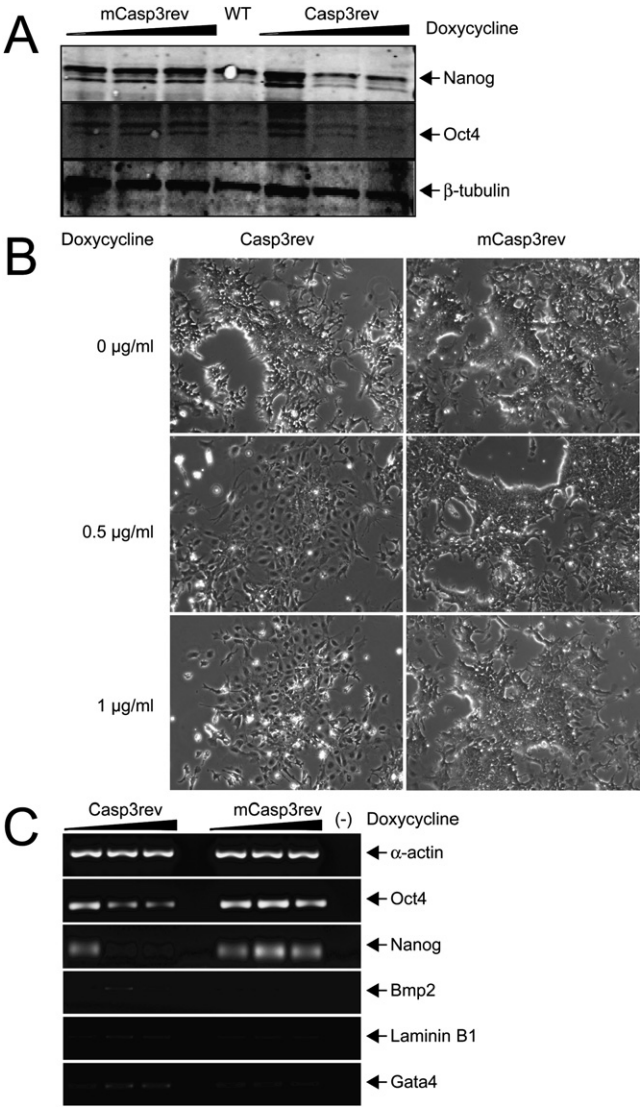

Figure 3. Ectopic Activation of Caspase-3 Activity in ESCs Leads to Differentiation

ESCs carrying a constitutively active form of *Casp3* gene (*Casp3rev*) or a mutated version (*mCasp3rev*) under the control of a tetracycline-inducible element were stimulated with doxycycline at increasing concentrations. (A) Western blotting and detection of Oct4 and Nanog were then performed. Both Oct4 and Nanog disappeared when the expression of *Casp3rev*, but not *mCasp3rev*, increased. WT, wild-type. (B) ESCs show clear morphologic signs of differentiation when *Casp3rev*, but not *mCasp3rev*, expression is increased. (C) RT-PCR of Oct4, Nanog, and differentiation markers. Both Oct4 and Nanog were downregulated when expression of *Casp3revI*, but not *mCasp3rev*, was increased, whereas expression of the differentiation markers Bmp2, Laminin B1, and Gata4 increased.

cells remain in a state of pluripotency or differentiate. To test this prediction, we again generated ESCs harboring cDNA coding for hNanog or D69E, the caspase cleavage-resistant form of hNanog targeted upstream of the *HPRT* locus under the control of a tetracycline-inducible element (Figures S7A and S7B). Importantly, the expression of induced hNanog reached levels comparable to those of endogenous Nanog (Figure S7C). ESCs expressing the D69E form of hNanog had a clear proliferative advantage over cells with the wild-type allele when cultured under conditions that promote differentiation (Figures 4E and 4F). They also lacked evidence of morphological changes, in contrast to ESCs carrying the wild-type form of Nanog (Figure S7D). To assess the antidifferentiation effects of caspase cleavage mNanog, we plated ESCs transfected with wild-type and D67G (cleavage-resistant) mNanog at clonal densities and analyzed colony formation 3 days later. The vast majority of colonies expressing the cleavage-resistant form of mNanog appeared morphologically unaffected and were positive for alkaline phosphatase, whereas a significantly lower number of colonies expressing wild-type mNanog consisted of undifferentiated, alkaline phosphatase-positive cells (Figure 4G). These observations support the hypothesis that caspase-mediated cleavage of Nanog promotes ESC differentiation.

DISCUSSION

Our findings suggest that ESCs exploit caspases for rapid and specific deactivation of Nanog, thus disrupting the autoregulatory circuit that otherwise preserves pluripotency in these cells. They indicate further that Caspase-3 plays a dominant role in this negative regulation by acting directly on Nanog or interacting as a cofactor with Caspase-9, which then deactivates the transcription factor. The action of caspases on Nanog appears to separate the N-terminal domain from the homeodomain, leading to the destabilization and subsequent degradation of the protein. From our experiments, it is clear that both human and mouse Nanog are cleaved by caspases, and therefore, it is likely that both of them are completely interchangeable with regard to caspase-mediated cleavage during the differentiation of ESCs. That the caspase cleavage-resistant forms of Nanog have a significantly stronger antidifferentiation effect in ESCs than does wild-type Nanog firmly suggests that caspase-mediated cleavage of endogenous Nanog plays a critical role in ESC differentiation.

The link we have identified between ESC differentiation and programmed cell death helps to explain several poorly understood observations on these ostensibly distinct processes, both in vitro and in the early embryo. For instance, some components of the cell-death system, such as Bcl-2, protect ESCs not only from apoptosis but also from differentiation (Yamane et al., 2005), while p53 has been shown to be directly involved in both the control of cell proliferation and apoptosis and the differentiation of ESCs (Lin et al., 2005). Moreover, other proteolytic components of ESCs, such as the proteasome, appear to have direct roles in the control of stem cell self-renewal (Szutorisz et al., 2006). Future studies will need to address the question of whether caspase activity indeed actively promotes differentiation, as our data suggest, or perhaps functions as part of a mechanism for the selective elimination of undifferentiated cells. In either case, the net outcome—cell differentiation—would be the same.

Selective targeting of Nanog by caspases is consistent with evidence implicating this transcription factor as a "master" regulator of the pluripotent state (Chambers et al., 2003; Ivanova et al., 2006; Mitsui et al., 2003). However, given the remarkable complexity of the pathways controlling cell death and differentiation, we find it difficult to imagine how Caspase-3, acting alone or in collaboration with Caspase-9, could be solely responsible

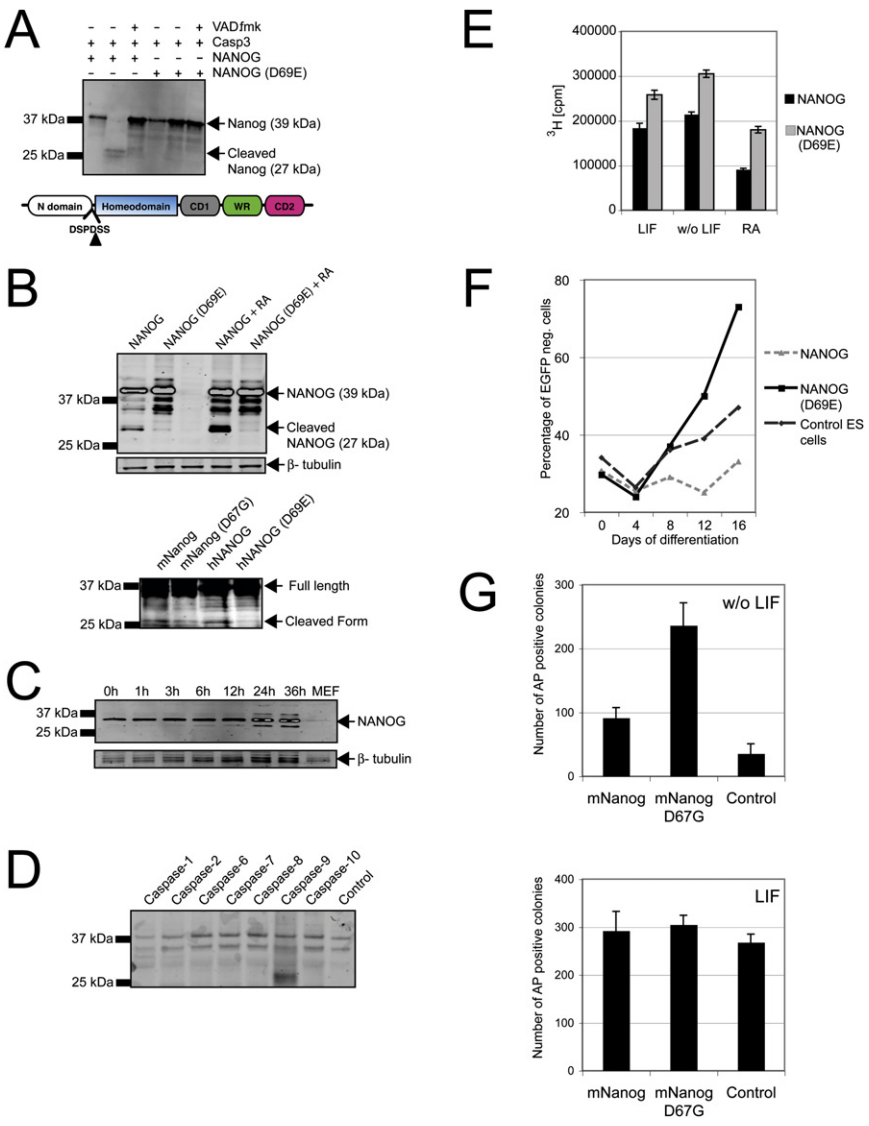

Figure 4. Caspases Cleave Nanog in ESCs

(A) Top: in vitro-translated hNanog, but not its D69E mutant, is cleaved by Caspase-3. Bottom: proposed caspase cleavage site at position 69, separating the N-terminal domain of Nanog from the rest of the protein.

(B) Top: western blot showing expression of hNanog and mutant hNanog with or without a mutation at position 69 (D69E) that obviates cleavage. The results represent ESCs transfected with hNanog or D69E hNanog in cultures treated with RA (1 μM) or untreated. After induction of differentiation, cleaved hNanog appeared as a 27 kDa band that was not visible when the cells were transfected with the mutated form of hNanog. Bottom: western blot showing expression of hNanog and mNanog and mutant hNanog and mNanog carrying a mutation at position 69 and 67, respectively (D69E and D67G), that obviates cleavage. The results represent ESCs transfected with depicted Nanog versions and treated with RA. Cleaved hNanog and mNanog appeared as a 27 kDa band that was barely visible when the cells were transfected with the mutated forms of hNanog and mNanog.

(C) Western blot analysis for endogenous mNanog reveals the cleaved form of the protein after induction of differentiation with RA.

(D) In vitro caspase cleavage assay reveals that Caspase-9 can effectively cleave in vitro-translated mNanog.

(E) Proliferation of ESCs harboring hNanog or D69E hNanog and grown under normal conditions with LIF or RA stimulation. Mean (±SD) levels of ^{3}H incorporation in triplicate experiments indicate a consistently greater proliferative advantage for cells expressing the hNanog cleavage mutant.

(F) Proliferation of ESCs expressing D69E or wild-type hNanog that were mixed 20:80 with EGFP-labeled ESCs. The percentage of unlabeled ESCs in the mixture was determined during growth in the absence of LIF. Cells carrying the caspase cleavage-resistant mutant (D69E hNanog) had a marked growth advantage over control or hNanog-expressing cells. The data are means ± SD of triplicate experiments.

(G) Mouse ESCs were plated at low densities (5000 cells/cm^2) and transfected with wild-type mNanog, D67G mNanog (caspase cleavage-resistant Nanog), or control plasmid (GFP) and cultured with or without LIF for 3 days. Tranfection with D67G mNanog substantially increased the number of undifferentiated (AP-positive) colonies.

for the mediation of proliferation versus differentiation choices in ESCs. We predict, instead, that other mediators of cell death participate in this critical developmental transition, possibly by targeting pluripotency factors other than Nanog. This hypothesis is supported by the absence of any overt phenotype in the preimplantation embryos of *Casp3* knockout mice (Kuida et al., 1996; Woo et al., 1998).

Our results leave unanswered the major question of why Caspase-3 activation during differentiation is limited and does not self-amplify as seen during apoptosis. Potential mechanisms for such control of caspase activity include subcellular localization of the enzyme, the caspase activity level itself, phosphorylation or other forms of structural modification, as well as direct or indirect interaction with protein inhibitors, such as IAPs. Given that ESCs carrying the cleavage-resistant form of Nanog proliferate better than those with the wild-type allele, it seems reasonable to suggest that the cleaved form of Nanog might exert a dominant-negative effect on some of the normal functions of Nanog.

A more comprehensive understanding of the molecular pathways controlling ESC self-renewal and differentiation, in particular the apparent molecular link between programmed cell death and cell differentiation, would not only accelerate efforts to generate clinically relevant cell types from ESCs but may also facilitate the reprogramming of differentiated cells to enter a pluripotent state (Okita et al., 2007), (Silva et al., 2006), for example, by blocking caspase activity. We consider the affinity of Caspase-3 and Caspase-9 for the transcription factor Nanog to be a paradigm for other potential caspase targets in ESCs; hence, it should be possible to exploit the caspases in experimental screens to identify other factors that regulate stem cell pluripotency. Finally, the involvement of caspases in nonapoptotic pathways, as

demonstrated here and elsewhere (Arama et al., 2003; De Botton et al., 2002; De Maria et al., 1999a, 1999b; Ishizaki et al., 1998), suggests that efforts to block apoptosis via caspase inhibition for therapeutic purposes may have much broader implications than initially thought, especially for stem cells.

EXPERIMENTAL PROCEDURES

ESC Cultures

The mouse ESC lines used were as follows: D3 (from ATCC, no. CRL11632), R1 (from Andras Nagy, Toronto), and E14Tg2a-derived A2lox (from Michael Kyba, UT Southwestern Medical Center) Cells were typically used between passages 10 and 20. A2lox ESCs express the reverse tetracycline transactivator from the endogenous *Rosa26* locus and carry an insertion containing a tetracycline response element, loxP-lox2272 sites and the neomycinΔ ATG-resistance gene upstream of *HPRT* on the X chromosome. To generate inducible derivatives, we subcloned cDNAs into the p2Lox targeting vector. Cre/Lox recombination was used to insert p2Lox into the inducible locus as described previously (Kyba et al., 2003). The medium contained 10% FBS (Innovative Research, cat. no. IFBF-H) tested for toxicity to ESCs in a colony-forming assay (Evans, 2004) that included Knockout DMEM (Invitrogen, cat. no. 10829-018), 1x nonessential amino acids (Invitrogen, cat. no. 111140-050), L-glutamine 1% v/v (Invitrogen, cat. no. 25030-081), 2-mercaptoethanol (3.5 μl per 250 ml medium; Sigma, cat. no. M7522), LIF 1000 per ml (Esgro, cat. no. ESG1107). Cells were routinely maintained on irradiated (8400 rad) mouse embryonic fibroblasts (MEFs) derived from E14.5-old embryos (mouse strain CF1, Charles River timed pregnant mice). For some experiments, ESCs were placed onto 0.1% gelatin type A (Sigma, cat. no. G1890)-coated dishes and passaged 2 to 4 times before the experiment was carried out. For differentiation studies, standard medium was replaced with medium containing 1 μM retinoic acid (RA, Sigma, cat. no. R2625) without LIF or N2B27 medium (Ying et al., 2003).

In Vitro Caspase Cleavage Assay

Trypsinized ESCs (R1, 200,000; D3, 250,000) were plated into 6 wells containing normal ESC medium, and on the next day, the medium was changed to 1 μM RA differentiation medium. Cells were harvested with trypsin-EDTA (Invitrogen, cat. no. 25300-054), and protein extracts were made in 0.1% CHAPS buffer, pH 7.2 (50 mM HEPES; 150 mM KCl; 1x protease inhibitors [Roche, cat. no. 1697498001]); 2 mM Na fluoride, and orthovanadate, respectively, at specified time points. The CaspGlo (Promega, cat. no. G8091) kit was used to directly measure caspase activity. Before determining caspase activity, we tested the system for variability within the dynamic range using recombinant caspase-3 (BD Bioscience, cat. no. 556471). The signal was measured with the Wallac 1429 VictorII instrument (Perkin-Elmer, Wellesley, MA).

Generation of Caspase-3 Knockout

ESCs were derived from E3.5-old mouse embryos. Briefly on day 1, caspase-3 homozygous (Kuida et al., 1996) and control C57/BL6 female mice were injected i.p. with 5 IU of PMS (Calbiochem, cat. no. 367222). Forty-six hours later, the female mice received an i.p. injection of 5 IU human chorionic gonadotropin (HCG; Calbiochem, cat. no. 230734). Stimulated females were placed into a cage with 8-week-old caspase-3-homozygous stud males. Blastocysts were collected on day 3 postcoitum in the afternoon. The blastocysts were placed onto irradiated mouse embryonic feeder cells and incubated for 3 days in ESC medium, trypsinized, and replated. Colonies apparent on day 7 were picked, expanded, and genotyped with use of the following primers: mCasp3-S, TGTCATCTCGCTCTGGTACG; mCasp3-AS, CCCTTTCTGCCTGTCTTCTG (PCR product SIZE 310 bp); Neomycin-S, AGACAATCGGCTGCTCTGAT; Neomycin-AS, ATACTTTCTCGGCAGGAGCA (PCR product size 260 bp); mOct4-S, GGCGTTCTCTTTGGAAAGGTGTTC; mOct4-AS, CTCGAACCACATCCTTCTCT (PCR product size 312 bp); Actin-S, GGCCCAGAGCAAGAGAGGTATCC, Actin-AS, ACGCACGATTTCCCTCTC AGC (PCR product size 460 bp). For PCR, we used GoTaq green master mix (Promega, cat. no. M7112) with the following program: 1 cycle at 95°C for 3 min followed by 40 cycles with 95°C for 30 s, 58°C for 30, and 72°C for 30 sec for 30 s followed by one extension of 10 min at 72°C. We generated two caspase-3 knockout cell lines (34A and 34B) and two control caspase-3 heterozygote cell line (22A and 22B). All experiments were carried out with these four cell lines and yielded essentially the same results.

Inducible Caspase-3 Cell Line Experiments

We used a constitutively active caspase-3 gene (*Casp3rev*) as described in Srinivasula et al., 1998 and a mutated version of *Casp3rev* (C1635) as described in Kamada et al., 1998. These active and inactive forms of caspase-3 were derived from the plasmids pGEMCasp3rev (6730 bp) and pGEM MutCas3rev (3867 bp). The caspase-3 inserts were cut out of their backbone vectors with EcoRI, and the insert (874 bp) was cloned by blunt end ligation into the pLoxP2 vector and digested with XhoI-SmaI (3549 bp). The vector was coelectroporated with pSalk-Cre into 30 × 10^6 A172 cells (960 μF, 25 millisec pulse). After electroporation, the cells were selected with G418 (400 μg/ml), and individual clones were picked and subsequently expanded. For the differentiation experiment, cells were plated into 6-well plates (1 × 10^5/well) and stimulated with 1 μg/ml doxycycline (Sigma, cat. no. D9891) for 36 hr. They were then harvested, and western blots for Oct4 (H-134 Santa Cruz, cat. no. SC9081), Nanog (Abcam, cat. no. AB14959; Chemicon 1/1000), and beta-tubulin (D10; Santa Cruz, cat. no. SC5274) were performed. Secondary antibodies (dilutions 1/5000 and 1/10000) consist of Alexa-680 antirabbit (Molecular Probes, cat. no. A21077) or IRday 800 antirabbit (Rockland, cat. no. 611-131-121). The signal was detected with the Odyssey system.

Detection of Nanog Cleavage by Caspase-3 In Vitro

Human Nanog was obtained from ATCC (cDNA clone cat. no. 10806397; Image Clone IDm no. 40004923; Gene Bank ID no. BC09827) and subcloned into pCR-BlendII-TOPO (Invitrogen). The mutation D69E was introduced into human hNanog with a site-directed mutagenesis kit (Quickchange II, Stratagene). Sequencing of both clones confirmed the identity of the human Nanog cDNA wild-type (TOPOT-Nanog) and D69E mutation (TOPO-mut -Nanog). In vitro transcription and translation were performed according to the manufacturer's protocol (Promega, cat. no. L520A). TOPO-Nanog and TOPO-D69E-Nanog plasmid 1 μg was incubated at 30°C with T7 quick master mix and 1 mM methionine plus biotin-tRNA (Promega, cat. no. L506A) in a total volume of 50 μl. The reaction was stopped after 90 min, and aliquots frozen at −80 C^0. Biotin-labeled human hNanog and D69E-Nanog were detected after transfer to nitrocellulose membranes with streptavidin Alexa fluor 680− (Molecular Probes, cat. no. S21378, dilution 1/10000). Protein was digested with 40 ng recombinant active caspase-3 (BD Bioscience, cat. no. 51-66281V) in a total volume of 15 μl at pH 7.5 (6 mM Tris-Cl, 1.2 mM $CaCl_2$, 1 mM KCl, 5 mM DTT, and 1.5 mM $MgCl_2$) (Laugwitz et al., 2001). The reaction product was run on 4%–20% gradient PAGE gels (Bio-Rad), and protein was detected as previously described (streptavidin, Alexa Fluor 680 Odyssey system, LI-COR).

Nanog Proliferation Assay

Nanog and D69E-Nanog clones were plated at a density of 5000 cells into 96-well plates, incubated overnight, and then stimulated with RA for specified times. Cells were then pulsed with methyl-^{3}H thymidine (Perkin Elmer, NET027) for 3 hr and harvested (Packard Filtermate harvester), and scintillation counts were measured with the Packard Topocount-NXT Microplate Scintillation and Luminescence Counter.

Nanog Competition Assay

The inducible cell lines Nanog, D69E mutated Nanog, EGFP, and control A172 were plated and incubated over night with 1 μM doxycycline. The Nanog inducible, D69E Nanog and A172 ESC lines were mixed with the EGFP inducible cell line in a ratio of 20:80 and plated on the next day. Cells were maintained in ESC medium without LIF. The percentage of EGFP-positive cells was determined with a FACScan instrument (Becton Dickinson) on days 0, 4, 8, 12, and 16 after plating.

SUPPLEMENTAL DATA

The Supplemental Data include seven figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/2/6/595/DC1/.

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