Supplemental Data

Caspase Activity Mediates the Differentiation of Embryonic Stem Cells

Jun Fujita, Ana M. Crane, Marlon K. Souza, Marion Dejosez, Michael Kyba, Richard A. Flavell, James A. Thomson, and Thomas P. Zwaka
Figure S1  Fujita et al.
Figure S1: Increased Caspase activity in ES cells upon induction of differentiation.

The D3 ES cell line was stimulated with retinoic acid (RA) or N2B27 differentiation medium for the indicated times. Caspase activity was measured with an *in vitro* assay (see Supplementary Methods). Mean (+/- SD) measurements from triplicate experiments are shown.
Figure S2  Fujita et al.
Figure S2. Caspase sensor system. (A) The caspase sensor contains a nuclear translocation signal (NLS), enhanced yellow fluorescent protein (EYFP), a 30-nucleotide-long sequence encoding the caspase cleavage site of PARP-1, and a cytoplasmic translocation signal (NES). This fragment was integrated upstream of the HPRT locus in 17-2lox ES cells via Cre-mediated recombination, placing it under the control of a tetracycline response element (TRE). The tet transactivator (rtTA) is targeted to the rosa26 locus on chromosome 6. (B) Western blot of protein lysates extracted from cells treated as indicated at the top of the graphic. Only doxycycline-treated cells showed expression of the reporter protein, which was cleaved only after induction of apoptosis.
Figure S3  Fujita et al.
Figure S3. Characterization of Casp3 knockout ES cell lines. (A) Genotyping PCR analysis showing that the knockout ES cell lines 34A and 34B contain only the knockout allele (smaller fragment), whereas the cell lines 22A and 22B contain both the larger wild-type allele and the smaller knockout allele. (B) Differentiation of Casp3+/− and Casp3−/− ES cell lines stimulated according to an embryoid body differentiation protocol. Although both cell lines initially formed similar embryoid bodies poststimulation (day 4), there were clear differences on 8 days. The Casp3−/− ES cells remained largely compacted and showed typical ES cell morphology, whereas Casp3+/− ES cells had differentiated completely. This difference had become even more pronounced by day 12. (C) Quantification of Oct4 activity by semiquantitative reverse transcriptase PCR. On days 8 and 10, Casp3−/− cells had more Oct4 signal than did Casp3+/− cells, indicating delayed differentiation. (D) Experiment similar to that shown in panel C but with real-time quantitative PCR. ΔCT versus GAPDH values. Again, Casp3−/− ES cells differentiated more slowly than Casp3+/− ES cells.
Figure S4  Fujita et al.
Figure S4: Details of teratoma formation experiment. Casp3+/− teratomas form typical structures such as neural epithelium (top) cartilage (middle) and endodermal structures (bottom), whereas Casp3−/− tumors are extremely homogenous and largely contain undifferentiated cell types.
Figure S5  Fujita et al.
**Figure S5. Inducible caspase-3 system.** (A) The constitutively active *Casp3* gene (*Casp3rev*) and a mutated version carrying a point mutation that renders the gene inactive (*mCasp3rev*) were subcloned (see Material and Methods). These fragments were then integrated upstream of the *HPRT* locus in A2Lox ES cells via Cre-mediated recombination, placing them under control of a tetracycline response element (TRE). The tet transactivator (rtTA) was targeted into the *Rosa26* locus on chromosome 6. (B) Western blot analysis of protein lysates extracted from two cell lines stimulated with increasing concentrations of doxycycline. Only doxycycline-treated cells carrying the Caspase-3 protein showed increased levels of active caspase-3. (C) Same two cell lines as shown in panels A and B were stimulated with doxycycline, and intrinsic caspase activity was measured with an *in vitro* Caspase activity assay (see Experimental Procedures). Values are means (+/- SD) of triplicate experiments.
Figure S6 Fujita et al.
Figure S6. mNanog is cleaved by caspases. (A) In vitro-translated mNanog is not cleaved by Caspase-3. (B) Caspase-3 activity assay (CaspGlo) reveals that both the hNanog caspase cleavage site peptide (DSPD) and the mNanog caspase cleavage site peptide (GSPD) effectively inhibit Caspase-3. (C) mNanog and mNanog (D67G) purified from ES cells by immunoprecipitation and exposed to recombinant Caspase-3 and Caspase-9. Only Caspase-9 effectively cleaved mNanog into a 27 kDa fragment. Mutation D67G in mNanog greatly diminished cleavage of mNanog by Caspase-9.
Figure S7 Fujita et al.
**Figure S7. Inducible hNanog expression by in ES cells.** (A) The hNanog gene and a mutated version carrying the point mutation D69E were subcloned. These fragments were integrated upstream of the HPRT locus in A2lox ES cells via Cre-mediated recombination, placing them under the control of a tetracycline response element (TRE). The tet transactivator (rtTA) is targeted to the Rosa26 locus on chromosome 6. (B) PCR analysis of RNA extracted from two cell lines stimulated with doxycycline. Only doxycycline-treated cells carrying wild-type or mutated hNanog showed increased levels of the transgene. (C) RT-PCR and simultaneous detection of endogenous Nanog and the hNanog transgene in cells stimulated with increasing concentrations of doxycycline. The highest concentration of doxycycline (1 µg/ml) results in a similar expression level of the hNanog transgenic as does the endogenous Nanog. (D) Phase-contrast, GFP channel and flow cytometry in mixing experiment carried out in Figure 4F. D69E hNanog samples showed a significant number of GFP-negative cells that grew in clusters with undifferentiated ES cell morphology, whereas most of the GFP-positive cells appeared to be differentiated.
Supplemental Experimental Procedures

Apoptosis rate

R1 ES cells (1 x 10^5) were plated into 6-cm dishes on irradiated MEFs and stimulated with RA differentiation medium for up to 72 hours. Cells were fixed with 4% paraformaldehyde (EMS, cat. no. 15710), stained with DAPI and mounted with Vectashield (Vector Lab, cat. no. H1200). The number of apoptotic figures was determined intriplicate per 100 cells. As a positive control, ES cells were incubated with staurosporine (Sigma, cat. no. S5921, 1μM) for 4 hours.

Caspase sensor

The pCasp3-sensor (Clontech) was used, and the sensor insert (950 bp) was amplified by using primers with SalI 5’ (gatgtcgactcagatccgctagccgcca) and Scal 3’ (gagtactttatctagatccggtggatcc) ends and cloned into the SalI-Smal cloning site of the p2Lox vector (Michael Kyba). P2LoxPcaspase3 sensor (20 µg) was co-electroporated with 20 ug of pSalk-Cre (Michael Kyba) into (1 x 10^6) A2lox ES cells (960 µF, 220 voltage, 25 millisec pulses). After electroporation, the cells were plated on neomycin-resistant MEFs (Chemicon, cat. no. PMEF-NL) and selected with G418 (400 µg/ml), individual clones were then picked and expanded. For the differentiation studies, a single clone was used (clone #6), 4000 cells/well were plated onto gelatine coated 8-chamber slides (Nalgen Nunc International) and stimulated with RA differentiation medium for specified times (cells incubated with staurosporine, 1 mM for 12 hours, served as a positive control). Cells were fixed with Cytofix (BD Biosciences, cat. no. 554714) at
room temperature for 30 minutes, washed 3 times with Perm/Wash™ buffer, incubated with anti-EGFP antibody (1:500, MBL), diluted in Perm/Wash™ buffer (BD Biosciences, cat. no. 554714) at 4°C overnight, washed 3 times with Perm/Wash™ buffer 3, and incubated with antirabbit antibody (Alexa Fluor 594, Molecular Probes, cat. no. A11072). They were mounted with VECTASHIELD plus DAPI. (Vector Labs). Cells that contained signal in the cytoplasm or in the nucleus were counted (100 cells per condition) and representative images were taken with the DeltaVision® deconvolution microscope (Applied Precision) equipped 40X objective lens. The acquired images were analyzed with softWoRx® Suite (Applied Precision).

**PARP-1 cleavage**

D3 ES cells (25 x 10⁴) were plated into gelatin-coated 6-well dishes and stimulated with RA. Cells were lysed with CHAPS buffer, and 20 µg of protein was loaded onto a PAGE gel and blotted. PARP-1 was detected with anti-PARP antibody (Santa Cruz). For PARP-1 immunostaining, R1 ES cells were plated onto 4-well chamber slides (Nunc, cat. no. 177437) and differentiation was induced with differentiation medium for 2 days. Cells were fixed for 30 minutes at RT in 4% paraformaldehyde (EMS, cat. no. 15710) in PBS and permeabilized for 5 minutes at room temperature (0.2% Triton X-100, Sigma, cat. no. T8532, in PBS). Blocking was performed with 5% goat serum for 24 hours at room temperature followed by incubation with the primary antibody anti-PARP-1-p85 (1/100 dilution, Promega, cat. no. G7341, (Perng et al., 2000). After 4 washes with 0.1% Tween20-PBS, cells were incubated with the secondary antibody (Abcam, cat. no. 6717, goat anti-FITC 1/500 in 0.1%Twin-20-PBS.), washed three times and mounted in
Active caspase 3 Western blot analysis

R1 ES cells were plated at a density of 2 x 10^5 cells per 6-well plate, kept in ES cell medium overnight and then stimulated for specified time with RA. Cells were lysed in CHAPS lysis buffer. Twenty µg of protein was run on a polyacrylamide gel and blotted onto nitrocellulose membrane (BioRad). Antibody incubation was carried out with the primary anti-active caspase-3 antibody (Cell Signaling, cat. no. 9661; 1/1000 dilution). The Rockland anti rabbit IgG antibody (dilution 1/5000 in PBS 0.1% Twin-20, IRDay800 611-132-122) served as a second antibody. Reactions were detected with the infrared Imaging System Odyssey (Li-COR protocol, doc. no. 988-07568).

VAD inhibition experiment

R1 ES cells were plated at a density of 2 x 10^5 cells/ml into 6-well plates containing ES cell medium. On the next day, medium was changed to RA differentiation medium with or without VAD (100 µM; Calbiochem, cat. no. 627610). Medium was changed every day and cells fixed at room temperature for 15 minutes in 4% paraformaldehyde (Polyscience, cat. no. 18814) followed by alkaline phosphatase staining according to the manufacturer’s instructions (Vector Laboratories, cat. no. SK5300 Alkphos Substrate III Blue.). Representative images were taken with a 10x objective. Colonies (1 x 10^2) were assessed for their morphology as follows: totally differentiated colonies = no AP staining, no ES cell morphology; mostly differentiated = colonies containing small areas
of undifferentiated AP-positive ES cells, but otherwise differentiated cells; partially
differentiated = colonies containing more than 50% undifferentiated, AP-positive cells;
undifferentiated = colonies lacking any discernible signs of differentiation.

**Teratoma, embryoid body formation and PCR for Oct4**

Caspase-3 heterozygous and homozygous ES cells were injected (2x10^6 cell in PBS) into
5 to 7 weeks old male NU/J mice (Charles River, stock no. 002019). Tumors that
eventually formed were extracted and subjected to histological analysis. For embryoid
body (E.B.) formation heterozygous and homozygous 2x10^6 ES cells were plated for 4
days in 60 mm plates, trypsinized, plated onto gelatin-coated plates and incubated in
differentiation medium. For Oct4 gene expression analysis, ES cells were harvested,
total RNA extracted (Qiagen, RNAeasy kit; cat. no. 74104) and reverse transcriptase
reactions (Imprint II reverse transcription system; Promega, cat. no. M7112) were carried
out according to the manufacturer’s instructions. Oct4 was amplified with the primers
shown in table 1 and GoTaq green master mix (Promega; cat. no. M7112) as follows: 1
cycle at 95°C for 3 minutes, followed by 25 cycles of 30 seconds at 95°C - 60°C - 72°C,
followed by one extension of 10 minutes at 72°C. In addition, quantitative PCR for Oct4
(cat. no. Mm0048129 gH part no. 433182) and control GAPDH (probe mouse 4352339E-
0611010) both from Applied Biosystems, were carried out with 2x TAQMAN Universal
master mix (Applied Biosystems). DeltaCT values were determined according to user
bulletin #2, ABI PRISM 7700, December 11, 1997). All experiments were carried out
with both heterozygous and homozygous ES cell lines and yielded essentially the same
results.
Detection of Nanog cleavage by endogenous caspase-3

Nanog and D69E-Nanog were cloned into the pEF1-luciferase-IRES-NEO vector (kindly provided by David Spencer, Baylor College of Medicine) by using XhoI and XbaI cloning sites, which together with a FLAG tag were added at the C-terminus by a PCR-based strategy with 5’-ggactcgagatgagtgtggatccagcttgtcc-3’ and 5’-tcctctagatcacttatcgtcatccttgtaatc-3’ primers. The plasmid was transfected into 293 cells and R1 ES cells with lipofectamine 2000 (Invitrogen). R1 ES cells (1 x 10⁵) were plated into 6-well plates and transfected on the following day with 10 µl lipofectamine 2000 plus (Invitrogen, cat. no. 52887) with 4 µg plasmid DNA. Differentiation was induced with RA differentiation medium. Proteins were extracted with CHAPS buffer with two cycles of freezing (dry-ice) for 30 minutes and sonication with 20 pulses. Twenty µg of protein was subjected to PAGE (12% Bio-Rad, 2 hours at 80V) and transferred with TG buffer (Bio-Rad) supplemented with 0.05% SDS and 20% methanol to 0.2 µm nitrocellulose membranes (Bio-Rad). Detection was carried out with the FLAG antibody M2 (Stratagene, cat. no. 200470-21) in a 1/10,000 dilution in blocking buffer (Li-COR, cat. no. 927-4000). Equal loading was confirmed by probinding for alpha-tubulin (antibody D10, Santa Cruz, SC5274).

Nanog-inducible ES cell line

Nanog and mutated D69E cDNAs were obtained by EcoRI digestion of TOPO-Nanog and TOPO-mut-Nanog plasmids, as described previously. The inserts (1263 bp each) were filled with Klenow and ligated to the p2LoxP vector which was cut with SmaI and XhoI and klenowed. The vector was co-electroporated with pSalk-Cre into A2lox ES
cells (960 µF, 220 millisec pulse, 15 x 10^6 cells). After electroporation, the cells were selected with 400 µg/ml of G418 (Invitrogen) and individual clones were picked, and subsequently expanded. Expression of inducible Nanog and inducible D69E Nanog was confirmed by western blotting and RT-PCR.