

Supplementary Material and methods

Reverse transcription-polymerase chain reaction

Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed with 1 µg of total RNA using the Superscript III first-strand cDNA synthesis kit (Invitrogen). The cDNA (0.5 µg) was used as a template in a mixture that contained Ampli Taq Gold polymerase (Applied Biosystems). Reverse transcription (RT)-polymerase chain reaction (PCR) was performed with an annealing temperature of 58°C. The primer sequences (forward and reverse) were as follows: Oct4, F: GGATGCTGTGAGCCAAGG, R: GAACAAAATGATGAGTGACAGACAG; Nanog, F: CACCCACCCATGCTAGTCTT, R: ACCCTCAAACCTCCTGGTCCT; p21, F: CCCTCTATTTTGGAGGGTTAATCT, R: GTACCCTGCATATACATTCCCTTC; p53, F: GATGACTGCCATGGAGGAGT, R: CTCGGGTGGCTCATAAGGTA; GAPDH, F: GGCATTGTGGAAGGGCTCA, R: TCCACCACCCTGTTGCTGT

Immunofluorescence

ESCs grown on glass coverslips were rinsed briefly with PBS and fixed for 20 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The ESCs were permeabilized for 10 min with 0.1% Triton X-100 in PBS, and blocked for 40 min with 4% BSA in PBS at room temperature. They were then incubated at 4°C overnight with the following antibodies: anti-Nanog (1:2000, Bethyl Laboratories), and anti-Oct-4 (1:200, sc-5279; Santa Cruz Biotechnology). This was followed by incubation with the following secondary antibodies: Alexa Fluor 488-labeled anti-mouse IgG, and Alexa Fluor 555-labeled anti-rabbit IgG (1:500;

Molecular Probes). Nuclei were counterstained with Hoechst 33342 (1 µg/mL) (Invitrogen). The slides were examined by confocal laser microscopy (C1si; Nikon, Tokyo, Japan) and the images were processed using the Nikon EZ-C1 viewer software.

Bromodeoxyuridine (*BrdU*) incorporation

The level of bromodeoxyuridine (BrdU) incorporation was measured together with the DNA content as previously described [19]. Briefly, ESCs 48 h after siRNA transfection were pulsed (45 min) with BrdU (1:100; Invitrogen). The ESCs were dispersed into single cells and then fixed overnight in 70% ethanol at 4°C. DNA denaturation was subsequently performed by incubation in 1 N HCl for 20 min at room temperature. The cells were then washed and incubated with 0.1 M sodium tetraborate for 10 min at room temperature. The cells were incubated with Alexa Fluor 488-conjugated mouse anti-BrdU antibody (1:100; Molecular Probes) in 2% BSA-PBS for 2 h at 4°C. The cells were then incubated with 100 µg/mL RNase (Boehringer Mannheim; Roche Applied Science, Basel Switzerland) for 15 min, followed by 40 min of incubation in freshly prepared propidium iodide (5 µg/mL). At least 10,000 cell events were recorded for each sample using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using the CellQuest program. The samples were subjected to two-parameter dot plot histogram analysis (BrdU incorporation vs. DNA content).