Up-Regulation of p27<sup>Kip1</sup> Protects hES Cells from Differentiation-Associated and Caspase 3-Dependent Apoptosis

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Recently, it has been suggested that p27<sup>Kip1</sup>, the cell cycle regulatory protein, plays a pivotal role in the progression of normal differentiation in murine embryonic stem (mES) cells. In the current study, we investigated the role of p27<sup>Kip1</sup> in the regulation of differentiation and apoptotic induction using Western blotting, quantitative real-time RT-PCR, and small interfering RNA (siRNA) assays and confocal laser scanning microscopic analysis of H9 human ES (hES) cells and H9-derived embryoid bodies (EBs) grown for 10 (EB<sub>10</sub>) and 20 days (EB<sub>20</sub>). Our results demonstrate that the proteins p27<sup>Kip1</sup> and cyclin D3 are strongly associated with cellular differentiation, and, for the first time, show that up-regulation of p27<sup>Kip1</sup> protects hES cells from inducing differentiation-associated and caspase 3-dependent apoptosis.

Keywords: p27<sup>Kip1</sup>, cyclin D3, apoptosis, caspase 3, embryoid bodies, differentiation

The capacity to replicate in a precisely regulated manner is a basic feature of life. This cellular process is regulated through the orchestrated mechanisms of the cell cycle that are governed by the cyclin-dependent kinases (CDKs) and specific CDK inhibitors (CKIs) [10, 15, 17, 19, 20]. Among the various CKIs, p27<sup>Kip1</sup> has been shown to play a crucial role in controlling the proliferation rate. It is well known, in general, that p27<sup>Kip1</sup> is a negative regulator of CDK2/cyclin E and CDK2/cyclin A, which drive cells from the G1 to the S phase of the cell cycle.

One of the fundamental features of embryonic stem (ES) cells is their unlimited robust proliferation with accurate differentiation potency. Therefore, ES cells differentiate and spontaneously form three-dimensional multicellular aggregates called embryoid bodies (EBs), if they are cultured in suspension without anti-differentiation factors. The differentiation of early embryonic cells is a precise and complex process by which highly proliferating, self-renewing ES cells switch their developmental status to differentiation. This critical event is usually accompanied by the reduction and/or halting of proliferation. Currently, it has been suggested that up-regulation of p27<sup>Kip1</sup> is responsible for the latter [2, 3, 9]. Furthermore, recent studies in murine ES (mES) cells provide some clues that p27<sup>Kip1</sup> also serves as a pivotal factor for regulating apoptosis in their differentiation. mES cells lacking p27<sup>Kip1</sup> undergo apoptosis before completing their differentiation [5, 6, 18]. In contrast, however, the potential role of p27<sup>Kip1</sup> in the transition from the undifferentiated to differentiated state of human (hES) cells has yet to be explored [1, 8]. Therefore, the current study was aimed at investigating the role of p27<sup>Kip1</sup> in the regulation of differentiation and apoptotic induction in NIH-registered H9 (WA09) hES cells and the H9-derived embryoid bodies EB<sub>10</sub> and EB<sub>20</sub>.

H9 hES cells were kindly provided by Prof. Kye-Seong Kim (College of Medicine, Hanyang University, Seoul, Korea). To generate H9-derived EBs, whole colonies of hES cells were detached with a glass pipette and cultured on tissue culture plates coated with Pluronic F-127 (Sigma, St. Louis, MO, USA) in medium without human recombinant basic fibroblast growth factor (Invitrogen, Carlsbad, CA, USA) for 10 (EB<sub>10</sub>) and 20 (EB<sub>20</sub>) days.

To examine the undifferentiated state of H9 hES cells, they were characterized using immunochemical staining for specific markers that are responsible for maintaining cell pluripotency and undifferentiated cellular phenotypes. As shown in Fig. 1, the undifferentiated state of hES cells was confirmed by specific detectable signals (red) for stage-specific embryonic antigen 4 (SSEA-4), tumor rejection antigen-I-60 (TRA-I-60), and TRA-I-81, as well as the

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The H9 cell line was examined using immunohistochemical staining for specific markers of undifferentiated hES cells. Alkaline phosphatase (AP) activity, and expressions of SSEA-4, TRA-1-60, and TRA-1-81 cell surface antigen on hES cells were visualized by fluorescence microscopy. However, the expression of SSEA-1 cell surface antigen was not detected. Labeled cells were viewed and photographed with a Nikon Eclipse TE2000-S phase-contrast microscope with fluorescence optics (fluorescent images, 10×). Bright-field microscopy demonstrated undifferentiated hES cells (top left).

Fig. 1. Characterization of H9 hES cells.

The presence of alkaline phosphatase (AP) activity. However, the expression of SSEA-1 cell surface antigen, a common marker for differentiated cells, was not detected. Moreover, lamin A/C expression activated during stem cell differentiation has been considered to be a novel marker for differentiated EBs [7]. As shown in Fig. 2A, Western blot analysis identified not only the absence of lamin A/C expression in hES cells but also its gradual up-regulation during the progression of differentiation in EBs.

To elucidate the molecular mechanism underlying differentiation of hES cells, differentiation-associated changes in protein levels were monitored in both undifferentiated hES cells and differentiated EBs in which differentiation was induced by withdrawal of LIF combined with RA treatment. As shown in Fig. 2A, the p27Kip1 protein level was only marginally detectable in undifferentiated hES cells but apparently increased following induction of differentiation in EBs and EBs. In addition, it is interesting to note that the expression patterns of individual D-type cyclin family members differed during the differentiation process in hES cells. In undifferentiated hES cells, cyclin D2 but neither cyclin D1 nor D3, was detectable. However, as early as day 10 of differentiation (EB0), cyclin D1 and D3 proteins were significantly up-regulated. Moreover, a significant decrease in cyclin D1 was identified in further differentiating EBs, whereas no change or an obvious increase in the level of cyclin D3 and D2, respectively, was found (Fig. 2A). Taken together, our data suggest that the differentiation-specific cyclins D3 and D1 could play crucial roles in the progression of normal differentiation of hES cells.

To examine whether increased p27Kip1 and cyclin D3 protein levels in differentiated EBs are due to the up-regulation of corresponding genes, semi-quantitative RTPCR was conducted in both undifferentiated hES cells and differentiated EBs. Real-time RT-PCR analyses were performed using an iCycler iQ Multi-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Specific primers were used for p27 (sense primer CTG CTGTGCTCCTCAAATACA, antisense primer GGTGTT TCTCAGGGGCTTCT), cyclin D3 (sense primer TTT GCCAGTAACCAGCCCACT, antisense primer CCCGCA GGCAGTCCACTTCA), and β-actin (sense primer GGC ATCCTCACACTTAAA, antisense primer GGGGTT TGGAGGTCTCTCAA). The relative quantization was calculated using the comparative threshold cycle method [13]. The housekeeping gene for β-actin was used to confirm the homogeneity of the DNA products.

As shown in Fig. 2B and 2C, the expressions of p27Kip1 and cyclin D3 in differentiated EBs were apparently up-regulated about 1.4- and 4.5-fold compared with that in undifferentiated hES cells, respectively. Recently, it has
been suggested that p27\(^{kip1}\) up-regulation could either be responsible for halting proliferation or regulating apoptosis in mES cell differentiation. In addition, down-regulation of D-type cyclins was also found to be associated with the induction of programmed cell death in many cell types [4, 11]. As such, the levels of both cyclin D2 and cyclin D3 were lowered dramatically in p27\(^{kip1}\)-deficient mES cells compared with their wild-type counterparts [6]. In this context, our results also demonstrate that p27\(^{kip1}\) and cyclin D3 are likely to serve as important factors in the differentiation of human embryonic cells, and also that p27\(^{kip1}\) is involved in maintaining appropriate levels of cyclin D1 and cyclin D3 for regulating differentiation-associated apoptosis.

To investigate the molecular mechanisms or pathway by which p27\(^{kip1}\) protects hES cells from differentiation-associated apoptosis, we performed knockdown of p27\(^{kip1}\) in EB\(_{10}\) cells using siRNA. Cells were transfected with either control siRNA (against fluorescein conjugate) or p27\(^{kip1}\)-siRNA, according to the manufacturer’s instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, the cells were collected and further analyzed using real-time RT-PCR and confocal microscopy.

After p27\(^{kip1}\)-siRNA treatment, about a 50% reduction in the p27\(^{kip1}\) expression level was obtained in EB\(_{10}\) cells compared with the control (Fig. 3A). Moreover, we found that p27\(^{kip1}\) knockdown had a considerable effect on apoptotic induction in EB\(_{10}\) cells, using microscopic examination, which showed significant morphological changes resembling apoptosis (data not shown). Recent studies have clearly demonstrated that apoptosis-inducing factor (AIF) activity is required for programmed cell death in mES cells. This AIF-dependent apoptosis does not involve the action of caspases. Therefore, it was inferred that caspase-independent apoptotic inductions are responsible for the massive cell death of differentiating p27\(^{-}\)-deficient mES cells, with the AIF-dependent pathway being the most likely candidate [6, 12].

To confirm whether a caspase-independent apoptotic pathway is also responsible for the death of differentiating hEB\(_{10}\) cells, we analyzed the activation of caspase 3 using confocal laser scanning microscopy. The control and p27-
siRNA-transfected EB<sub>10</sub> cells were fixed with BD Cytofix/Cytoperm kit buffer (Becton Dickinson, San Jose, CA, USA) and then washed twice with BD Perm/Wash<sup>TM</sup> kit buffer (Becton Dickinson). DNAs were stained for 5 min using 10 μg/ml DAPI and washed twice with PBS. Incubation with caspase 3 antibody (Cell Signaling Technology, Beverly, MA, USA) was performed for 4 h at room temperature and visualized with donkey anti-rabbit IgG FITC antibody (Jackson ImmunoResearch, West Grove, PA, USA). Images were obtained using confocal laser scanning microscopy (Olympus FV1000; Tokyo, Japan).

Interestingly, our data indicate that activation of caspase 3 was required for differentiation-associated apoptotic induction via knockdown of p27<Kip1> in EB<sub>10</sub> cells. As shown in Fig. 3B, active caspase 3 (green) was observed in the cytoplasm of EB<sub>10</sub> cells treated with p27<sup>Kip1</sup>-siRNA, whereas it was not observed in control EB<sub>10</sub> cells.

The up-regulation of p27<sup>Kip1</sup> in cancer cells, in general, is typically associated with induction of cell cycle arrest and apoptosis [14, 16]. In contrast, down-regulation of p27<sup>Kip1</sup> by specific antisense oligonucleotides leads to apoptotic induction of differentiating cells [2, 6]. Therefore, cell type-specific functioning of p27<sup>Kip1</sup> may well explain the occurrence of both pro- and anti-apoptotic effects of p27<sup>Kip1</sup> in differentiating cells.

In conclusion, we suggest that up-regulation of p27<sup>Kip1</sup> is at least in part responsible for preventing hES cells from differentiation-associated and caspase 3-dependent apoptotic induction, whereas AIF-dependent and caspase-independent apoptotic inductions are responsible for the massive cell death of differentiating p27<sup>Kip1</sup>-deficient mES cells. Unfortunately, the current study does not provide a specific molecular mechanism underlying the anti-apoptotic activity of p27<sup>Kip1</sup> and cyclin D3 in differentiation of cells of human embryonic origin. Therefore, further studies should be performed to elucidate whether up-regulation of p27<sup>Kip1</sup> plays a pivotal role in maintaining a proper level of cyclin D3 for its anti-apoptotic effect on the normal differentiation of hES cells to occur.

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