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Bcl-xL enhances single-cell survival and expansion of human embryonic stem cells without affecting self-renewal

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Abstract Robust expansion and genetic manipulation of human embryonic stem cells (hESCs) and induced-pluripotent stem (iPS) cells are limited by poor cell survival after enzymatic dissociation into single cells. Although inhibition of apoptosis is implicated for the single-cell survival of hESCs, the protective role of attenuation of apoptosis in hESC survival has not been elucidated. Bcl-xL is one of several anti-apoptotic proteins, which are members of the Bcl-2 family of proteins. Using an inducible system, we ectopically expressed Bcl-xL gene in hESCs, and found a significant increase of hESC colonies in the single-cell suspension cultures. Overexpression of Bcl-xL in hESCs decreased apoptotic caspase-3$^+$ cells, suggesting attenuation of apoptosis in hESCs. Without altering the kinetics of pluripotent gene expression, the efficiency to generate embryoid bodies (EBs) in vitro and the formation of teratoma in vivo were significantly increased in Bcl-xL-overexpressing hESCs after single-cell dissociation. Interestingly, the number and size of hESC colonies from cluster cultures were not affected by Bcl-xL overexpression. Several genes of extracellular matrix and adhesion molecules were upregulated by Bcl-xL in hESCs without single-cell dissociation, suggesting that Bcl-xL regulates adhesion molecular expression independent of cell dissociation. In addition, the gene expressions of FAS and several TNF signaling mediators were downregulated by Bcl-xL.
These data support a model in which Bcl-xL promotes cell survival and increases cloning efficiency of dissociated hESCs without altering hESC self-renewal by i) attenuation of apoptosis, and ii) upregulation of adhesion molecules to facilitate cell–cell or cell–matrix interactions.

Introduction

Pluripotent stem cells, including human embryonic stem cells (hESCs) and induced-pluripotent stem (iPS) cells, are capable of self-renewal and multilineage differentiation.

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Pluripotent stem cells not only have enormous potential as a source of therapeutic tissues, but also provide a unique system for studying lineage commitment and early human development (Murry and Keller, 2008; Passier et al., 2008). Due to low survival rate as single cells, hESCs are commonly grown as small clusters after collagenase treatment following mechanical scraping, resulting in limited expansion of hESCs (Amit et al., 2000). Enhancement of hESC survival is a critical step for rapid hESC expansion and lineage differentiation. Recent studies demonstrated that Y-27632, a specific inhibitor for Rho-dependent protein kinase (ROCK), improves hESC survival by blocking dissociation-induced cell death (Watanabe et al., 2007; Ohgushi et al., 2010; Chen et al., 2010). Other small molecules that inhibit the Rho-ROCK pathway also enhance hESC survival (Xu et al., 2010).

Spontaneous differentiation of hESCs into different cell types can be triggered by formation of 3-dimensional (3D) embryoid bodies (EBs) (Itskovitz-Eldor et al., 2000). Although the EB is far less organized than an embryo, it can partially mimic the spatial organization of cells in an embryo (Doetschman et al., 1985), allowing the assessment of cell–cell interactions and the developmental niche in vitro. However, the formation of EBs from hESCs is inefficient because of low survival of hESCs, and usually requires an entire colony of hESCs, resulting in variable sizes of EBs, thus rendering poor reproducibility of the differentiation procedure. We and others have developed systems to induce hESC differentiation directly for investigating the roles of extracellular molecules in lineage-specific differentiation (Bai et al., 2010; Wang et al., 2007; Li et al., 2011; Yu et al., 2011; Zhu et al., 2009). However, we were unable to use direct differentiation of hESCs to assess the effect of cell–cell interaction during hESC differentiation.

The assumption that apoptosis is involved in hESC single-cell survival is plausible. Diverse groups of molecules are involved in the apoptotic pathway. One set of mediators functioning in apoptosis are asparate-specific cysteine proteases or caspases. Sequential activation of caspase cascades has a pivotal role in the execution-phase of cell apoptosis. Wang X et al. (2009) recently reported that inhibition of caspase-mediated anoikis is critical for FGF2-sustained culture of hESCs and iPS cells. The B-cell lymphoma-2 (Bcl-2) family, consisting of 25 pro- and anti-apoptotic members, regulates a caspase apoptotic cascade (Swanton et al., 1999) and maintains a balance between newly-formed cells and old, dying cells (Reed and Pellecchia, 2005). When anti-apoptotic Bcl-2 family members are overexpressed, the ratio of pro- and anti-apoptotic Bcl-2 family members is disrupted and apoptotic cell death can be prevented (Kang and Reynolds, 2009). Mouse ES cells overexpressing Bcl-2 proliferate in serum-free and feeder-free conditions when supplemented with LIF (Yamane et al., 2005), indicating that attenuation of apoptosis is critical for ES cell survival and self-renewal. An anti-apoptotic protein of the Bcl-2 family, Bcl-xL, contains all four Bcl-2 homology domains (Reed and Pellecchia, 2005; Packham and Stevenson, 2005).

Bcl-2 and Bcl-xL are expressed in undifferentiated hESCs and differentiating EBs (Wang et al., 2009; Rho et al., 2006). To improve the efficiency of hESC growth and differentiation, we investigated the protective role of Bcl-xL in dissociation-induced hESC death. Here, we demonstrated that activated caspase-3$^+$ apoptotic cells, as well as gene expression of other apoptotic-related genes, were significantly increased when hESCs were dissociated into single cells. Ectopic expression of Bcl-xL prevented hESCs from undergoing apoptosis following enzymatic dissociation into single cells, resulting in both an increase of hESC colonies and an increase of differentiation efficiency to form EBs. However, hESC self-renewal was not altered by overexpression of Bcl-xL. Our study demonstrated that Bcl-xL overexpression not only decreased apoptotic caspase-3$^+$ cells, but also downregulated pro-apoptotic TNF signaling mediators. In addition, Bcl-xL regulated gene expression of adhesion molecules, suggesting an enhancement of attachment and cloning efficiency of single hESCs.

Results

Rapid increase of caspase-3$^+$ apoptotic cells after hESC dissociation

One limiting factor for hESC and iPS cell expansion is poor cell survival during subcultures. To verify that hESCs underwent apoptosis after enzymatic dissociation, we assessed apoptotic onset at different time points after hESC dissociation into single-cells. Caspase-3 acts as a key mediator of apoptosis in mammalian cells, and activation of caspase-3 is one of the penultimate steps in apoptotic cell death pathways (Kothakota et al., 1997; Pirnia et al., 2002; Fischer et al., 2003). We used specific antibodies for the subunit of cleaved caspase-3 (activated caspase-3) to determine caspase-3 activation following enzymatic dissociation of hESCs (Fig. 1A). Flow cytometry has been used to quantify the apoptotic cells containing activated caspase-3 (Garza et al., 2009; Lawson et al., 2004). Our data of flow cytometry indicated that the caspase-3$^+$ population rapidly increased following enzymatic dissociation of hESCs (Fig. 1B). Approximately 18% of the cells were caspase-3$^+$ in the first 3 h, whereas a moderate increase of caspase-3$^+$ cells was observed between 3 and 6 h. Concurrently, the number of the non-viable cells, which stained for 7-AAD, increased steadily over time (Supplementary Fig. 1). Parallel analysis by quantitative PCR (qPCR) showed that after hESC dissociation into single cells, the expressions of anti-apoptotic genes, such as Bcl-2A1 and Bcl-xL, were downregulated; whereas, the expressions of several pro-apoptotic related-genes, including tumor necrosis factor receptor (TNFR) superfamily member-9 (TNFRSF9), tumor necrosis factor (TNF) superfamily member 8 (TNFSF8), and TNF ligand family member LTA, were upregulated (Fig. 1C). However, qPCR array analysis indicated that trancription of the caspase genes was not affected in dissociated hESCs (Supplementary Fig. 2). These data demonstrated that (i) hESC-dissociation induced rapid and extensive apoptotic response in hESCs, thereby leading to subsequent cell death, and (ii) the caspase-3 activity in dissociated hESCs was regulated at the post-transcriptional level.

Attenuation of apoptosis by overexpression of Bcl-xL in hESCs

The caspase cascade is mediated by the Bcl-2 family of proteins in mitochondria-dependent apoptosis (Swanton

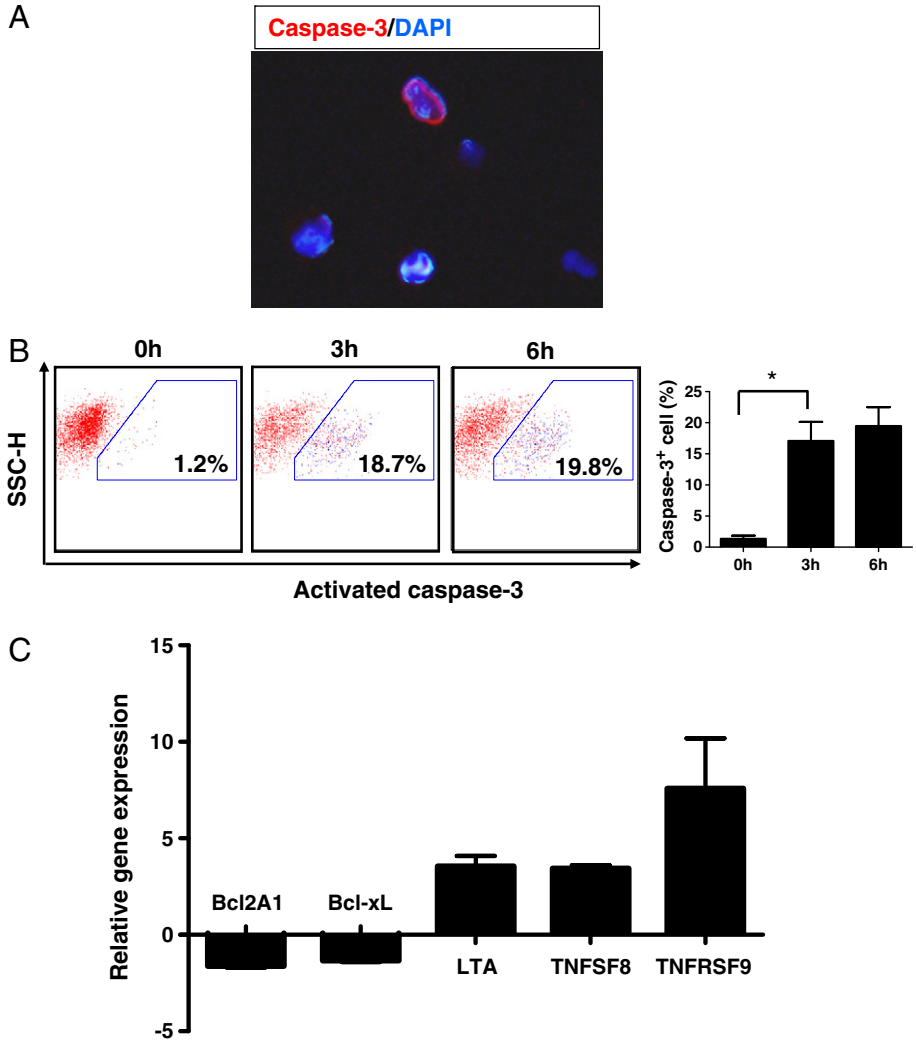

Figure 1 Increase of apoptotic cells after hESC dissociation into single cells. HESCs were treated by Accutase to generate single-cell suspensions. (A) Activated caspase-3 was detected by immunostaining after 3 h of hESC dissociation. Cells were collected on a glass slide by cytospinning to take photos under a fluorescence microscope. (B) Apoptotic cells, in which caspase-3 is activated, were analyzed by flow cytometry at different time points after hESC dissociation into single cells. Data are representative of three independent experiments. *$p<0.05$ is considered statistically significant. (C) After 6 h of hESC dissociation into single cells, the expressions of the pro-apoptotic genes (LTA, TNFSF8 and TNFRSF9) and the anti-apoptotic genes (Bcl2A1 and Bcl-xL) were analyzed by qPCR. A single-cell suspension at 0 h was used as a control.

et al., 1999). We next investigated whether attenuation of apoptosis by ectopic expression of Bcl-xL in an inducible lentiviral system enhances hESC survival. Expression of the human Bcl-xL gene was controlled by a tetracycline inducible promoter in the lentiviral vector pLentiGFPtc, and GFP expression was driven by the human EF-1alpha promoter (Fig. 2A). Bcl-xL-expressing hESCs (H1-Bcl-xL) and vector control hESCs (H1-GFP) were established after several runs of manual selection of GFP^+ hESC colonies. Without doxycycline induction, Bcl-xL was expressed at base levels in hESCs. Bcl-xL expression in H1-Bcl-xL hESCs was induced by doxycycline in a dose-dependent manner (Fig. 2B).

To test the anti-apoptotic effect of Bcl-xL upon hESC dissociation, we measured caspase-3 activity in H1-Bcl-xL hESCs by flow cytometry. Compared with H1-GFP control cells, the number of caspase-3^+ cells was decreased in H1-Bcl-xL hESCs upon doxycycline induction (Fig. 2C). However, transcription of the caspase genes was not altered by Bcl-xL expression before (0 h) and after (6 h) hESC dissociation (Supplementary Fig. 3), suggesting that caspase-3 activity triggered by single-cell dissociation are regulated at the post-transcriptional level in Bcl-xL-expressing hESCs. It is unclear whether the anti-apoptotic function of Bcl-xL in hESCs is mediated specifically through inhibition of the pro-apoptotic effects of caspase-3.

Bcl-xL increased hESC single-cell cloning efficiency without affecting self-renewal

HESCs in single-cell culture have poor survival rates, resulting in fewer colonies than hESCs from small clusters (Amit et al., 2000; Hasegawa et al., 2006). To test whether

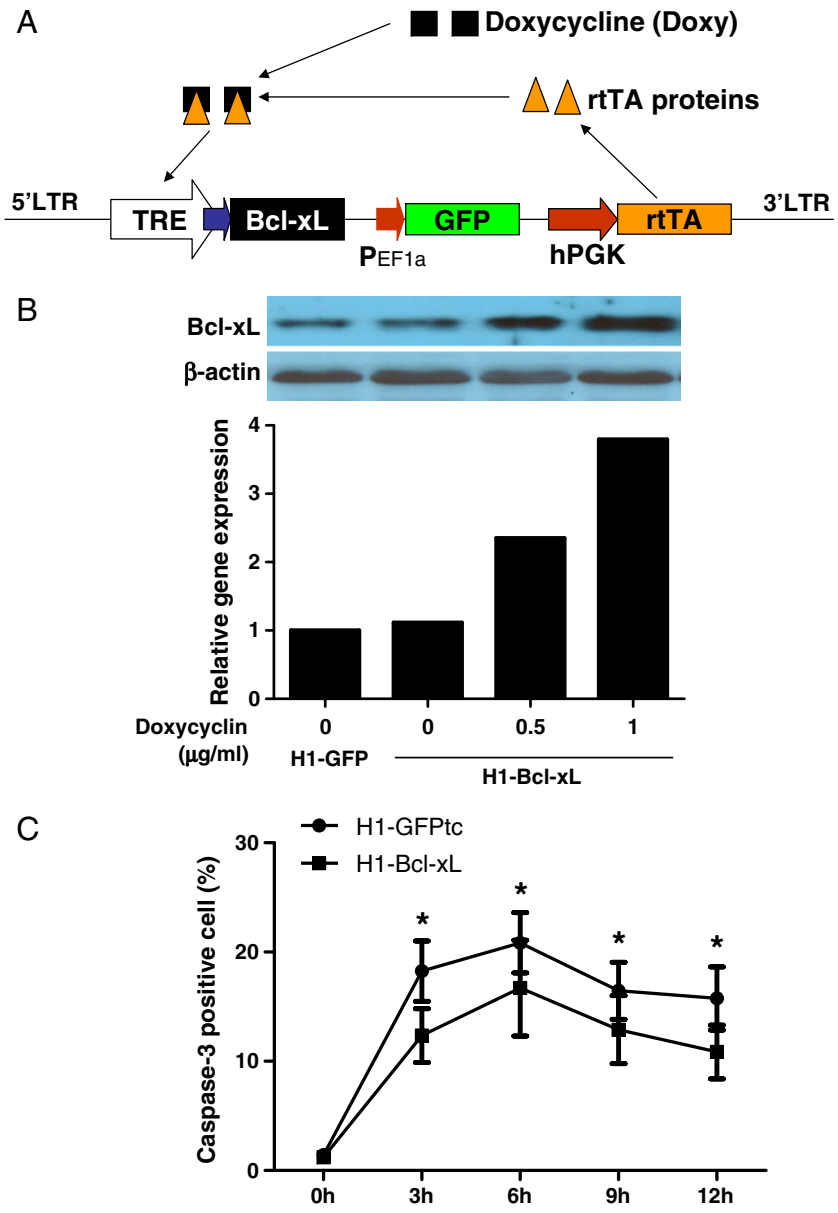

Figure 2 Attenuation of apoptosis by Bcl-xL overexpression in hESCs. (A) Schematic diagram of inducible strategy in a lentiviral vector. The human Bcl-xL gene was driven by a tetracycline inducible promoter. The constitutive expression of GFP was driven by a human EF-1alpha promoter. (B) Western blot analysis of Bcl-xL in H1-Bcl-xL cells. Different concentrations of doxycycline were tested to induce Bcl-xL expression in the H1-Bcl-xL cells. H1-GFP cells were used as control hESCs. (C) Decrease of apoptotic cells in H1-Bcl-xL cells. HESCs were dissociated into single cells. Activated caspase-3$^+$ cells in the presence of doxycycline (500 ng/ml) were determined by FACS every 3 h. Data are representative of three independent experiments. *$p<0.05$ is considered statistically significant.

overexpression of Bcl-xL enhances single-cell survival, we cultured single-cell suspension of hESCs on MEF feeder cells or Matrigel-coated wells, and determined hESC colony numbers with or without Bcl-xL ectopic expression. Compared with the H1-GFP control, the numbers of hESC colonies increased significantly in H1-Bcl-xL cells upon induction of Bcl-xL expression (Figs. 3A and B). Culture on MEF feeder cells gave rise to more hESC colonies (Fig. 3A) than those on Matrigel-coated wells (Fig. 3B). However, the sizes of hESC colonies were similar with or without doxycycline induction of Bcl-xL expression (Supplementary Fig. 4), suggesting that Bcl-xL increased hESC single-cell cloning efficiency without affecting self-renewal. After 6 days of culture, the average cell number per colony of H1-Bcl-xL cells was approximately 500 cells with or without doxycycline induction (Supplementary Fig. 4).

The self-renewal and survival of hESCs may be mediated by para/autocrine signals (Pyle et al., 2006). To test whether hESCs overexpressing Bcl-xL provide paracrine signals for cell growth, we mixed GFP$^+$ H1-Bcl-xL cells (30%) with GFP$^-$ parent hESCs (70%). The ratio of H1-Bcl-xL cells (GFP$^+$ colonies) versus parent hESCs (GFP$^-$ colonies) was measured in the subsequent culture. As shown in Fig. 3C, the ratio of GFP$^+$ versus GFP$^-$ colonies increased to approximately 60%

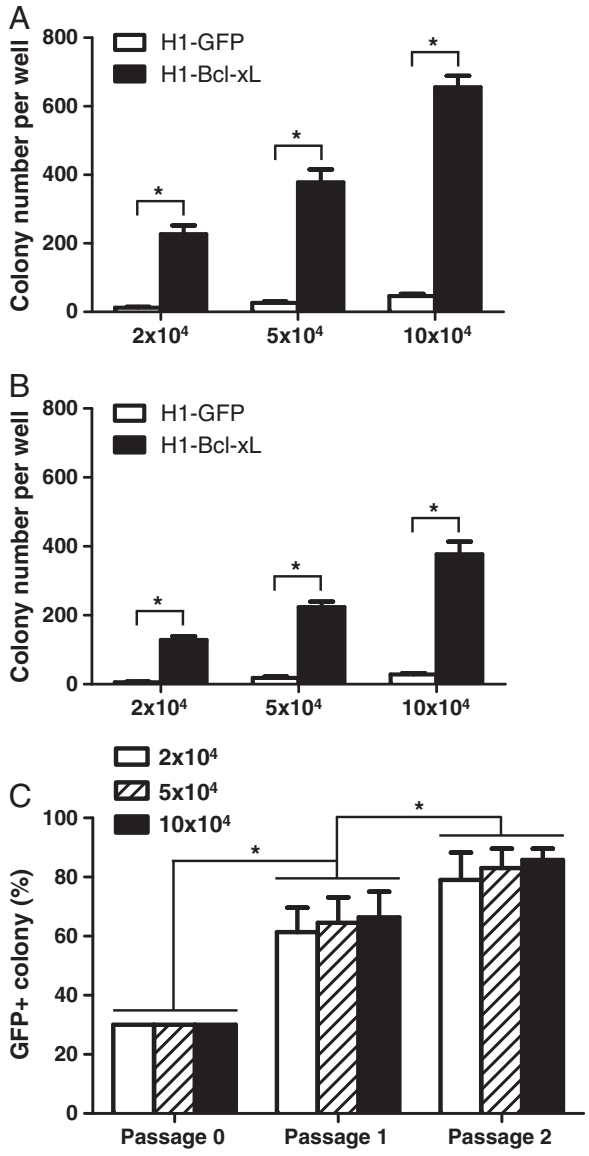

Figure 3 Increase of single-cell survival by overexpression of Bcl-xL in hESCs. Single-cell suspensions of hESCs (2, 5, or 10×10^4 cells per well) were subcultured on MEF (A) or on Matrigel-coated wells (B) in 12-well plates in hESC growth medium in the presence of doxycycline (500 ng/ml). The hESC colony numbers in either MEF (A) or Matrigel-coated wells (B) were counted from triplicate wells after six days of culture. *$p<0.05$ is considered statistically significant. (C) Competitive growth of H1-Bcl-xL cells (30%) and parent hESCs. The mixture of single-cell suspensions of H1-Bcl-xL cells (30%) and parent hESCs (70%) was cultured on MEF for six days. The hESC colonies were counted under a fluorescence microscope after the first and second passage. The percentage of GFP^+ hESC colonies was calculated for the frequencies of H1-Bcl-xL colonies.

and 80% after one and two subcultures, respectively. Similar elevation of GFP^+ versus GFP^- colonies was observed in the cultures at low, medium or high cell density (2×10^4, 5×10^4, or 10×10^4 cells) (Fig. 3C), indicating that cell density had no significant effect on the ratio of GFP^+ versus GFP^- colonies. Our study suggested that overexpression of Bcl-xL in hESCs increases single-cell survival during hESC growth in a paracrine signal-independent manner.

To determine whether overexpression of Bcl-xL affects hESC pluripotency, we examined pluripotent gene expression in H1-Bcl-xL cells that were cultured for 6 days with doxycycline induction. Immunohistochemistry and flow cytometric analysis showed that hESC pluripotent markers, including SSEA-4, TRA-1-60, and TRA-1-81, were expressed in undifferentiated H1-Bcl-xL cells with or without doxycycline induction (Fig. 4A), similar to the behavior of the parent hESCs and H1-GFP control cells (data not shown). To examine whether Bcl-xL alters the kinetics of pluripotent gene expression during hESC differentiation, we induced hESC differentiation in EBs for 21 days in the presence of doxycycline. RT-PCR analysis at different time points showed that Oct4 and Nanog expression patterns were similar in H1-Bcl-xL cells and H1-GFP cells (Fig. 4B). This result was further confirmed by qPCR (Fig. 4C). Our data suggested that the kinetics of pluripotent gene expression is not altered by Bcl-xL overexpression during hESC differentiation.

To determine whether ectopic expression of Bcl-xL affects hESC proliferation, we cultured H1-Bcl-xL hESCs as small clusters. In contrast to the result observed with hESC cultures initiated with single cells, overexpression of Bcl-xL had no significant effect on hESC colony number (Fig. 4D) and size (Fig. 4A) when H1-Bcl-xL cells were subcultured as clusters. The growth potential of H1-Bcl-xL hESCs that were cultured as clusters was not significantly different from H1-GFP control cells at passages 5, 15, and 25 (Fig. 4D). Our data suggest that Bcl-xL increases clonal survival of dissociated hESCs by enhancing the attachment and survival of single hESCs.

Overexpression of Bcl-xL increased the efficiency of EB formation in vitro and teratomas in vivo

Differentiation of hESCs is conventionally induced from large hESC colonies to circumvent the restriction of low EB formation efficiency after single-cell dissociation (Amit et al., 2000; Hasegawa et al., 2006). As a consequence, the resulting EBs vary in sizes, making it difficult to control hESC differentiation. To examine the effect of Bcl-xL on the efficiency of EB formation, we employed the hanging-drop method with defined cell numbers to generate uniform EBs. Compared to H1-GFP control cells, the efficiency of EB formation increased significantly in H1-Bcl-xL cells grown under Bcl-xL induction conditions (Fig. 5A). When 500 cells in each drop were used, approximately 40% of the drops formed EBs in H1-Bcl-xL cells, compared to approximately 5% of the EB containing drops from H1-GFP control cells. When 1000 cells per drop were used to form EBs, approximately 60% of the drops contained EBs from H1-Bcl-xL cells, compared to approximately 15% EB containing drops from H1-GFP cells. Further increase of cell numbers up to 2000 cells had a modest effect on EB formation from either H1-Bcl-xL cells or H1-GFP cells (data not shown). The qPCR analysis indicates that PAX6 and MAP2 gene expressions during hESC differentiation by Bcl-xL overexpression were upregulated, whereas RUNX1, PITX and FOXA2 gene expressions were downregulated (Supplementary Fig. 5).

We further examined whether Bcl-xL expression affects teratoma formation in nude mice. As shown in Fig. 5B, tissues derived from three germ layers including gland, cartilage, and neural cells, were observed in teratomas that originated from H1-Bcl-xL hESCs, suggesting that H1-Bcl-xL hESCs remain pluripotency. Interestingly, the teratomas generated from Bcl-xL overexpressing cells were significantly larger than those from H1-GFP control cells (Fig. 5C), suggesting that Bcl-xL enhances hESC survival and growth in vivo.

Upregulation of adhesion molecules and downregulation of TNF signaling mediators by Bcl-xL

Adhesive interactions between cells–cells and cells–extracellular matrix proteins (ECM) are critical to many biological processes, including cell proliferation and cell survival (Schwartz and Ginsberg, 2002; Kumar, 1998; Martin et al., 2002). Adhesion molecules, such as EpCAM and E-cadherin, are

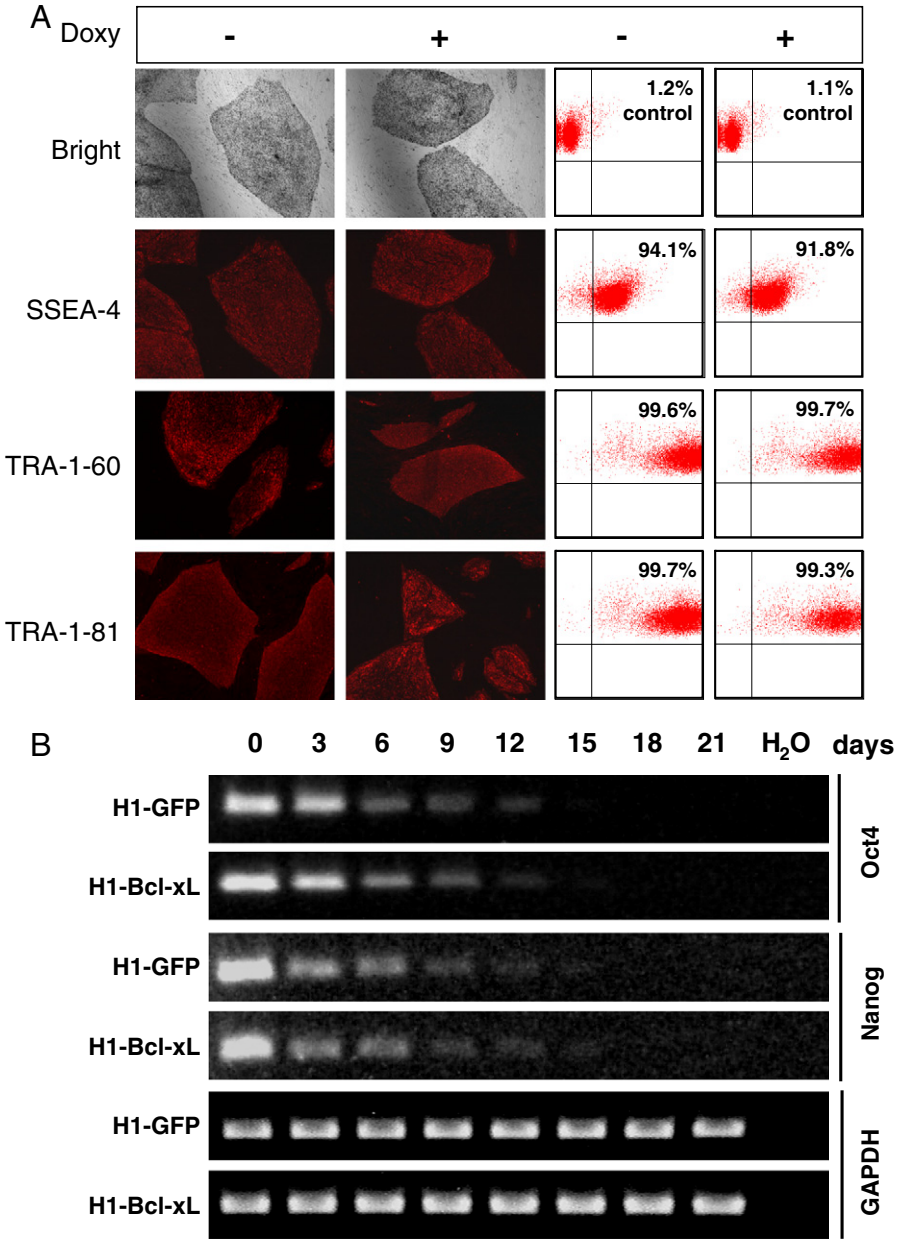

Figure 4 Pluripotency of H1-Bcl-xL hESCs. (A) Analyses of immunohistochemistry and flow cytometry for pluripotent genes, SSEA-4, TRA-1-60, and TRA-1-81. H1-Bcl-xL cells were cultured with or without doxycycline (500 ng/ml) 6 days prior to immunostaining. Flow data are representative of three independent experiments. (B) RT-PCR analysis of pluripotent genes during hESC differentiation. H1-Bcl-xL hESC colonies were differentiated to form EBs in the presence of doxycycline (500 ng/ml) on ultra-low attachment plates. RNA samples were harvested at different time points for RT-PCR analysis. The H1-GFP cells were used as a control. (C) The gene expressions of OCT4, NANOG, and SOX2 were analyzed by qPCR. Undifferentiated hESCs were used as controls. (D) The effect of Bcl-xL on hESC growth. H1-Bcl-xL hESCs and H1-GFP hESCs were subcultured from small clusters after collagenase treatment. The hESC colony numbers were counted after 6 days of hESC culture in the presence of doxycycline.

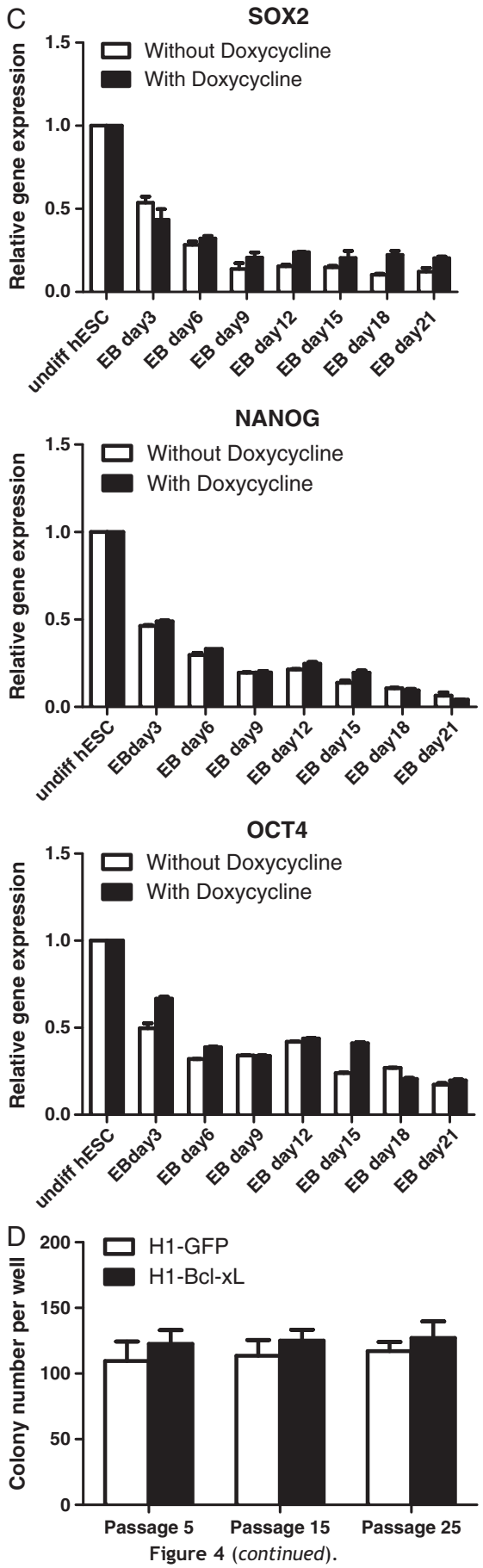

Figure 4 (*continued*).

involved in maintenance of murine and human embryonic stem cell phenotypes (Xu et al., 2010; Ng et al., 2010; Gonzalez et al., 2009). To investigate the potential adhesive interaction involved in hESC survival, we analyzed gene expression of adhesion molecules in H1-Bcl-xL hESCs. By analyzing the expression profile of 84 adhesion molecules using a qPCR array, we found that 18 of these adhesion molecule genes were upregulated by more than a two-fold increase in H1-Bcl-xL hESCs (Supplementary Table 3). The upregulation of extracellular matrix protein 1 (ECM1), fibronectin 1 (FN1), CD44, integrin-α3 (ITGA3), collagen VI-α2 (COL6A2), thrombospondin 1 (THBS1), and TIMP inhibitor 1 (TIMP1) was confirmed by qPCR (Fig. 6A). Upregulation of adhesion molecules by Bcl-xL expression suggests that Bcl-xL may promote hESC survival in part by enhancing the hESC adhesion potential to feeder cells or Matrigel. Consistent with a previous study, E-cadherin transcripts were not altered during hESC dissociation (Xu et al., 2010). The functional roles of individual adhesion molecules are currently under investigation.

To gain more insight into the apoptotic status, we next analyzed the expression of pro-apoptotic-related genes by qPCR array. Several members of TNF-related ligands and receptors that play important roles in regulating apoptosis (Schulze-Osthoff et al., 1998) were downregulated in H1-Bcl-xL hESCs before (0 h) and after (6 h) hESC dissociation (Figs. 6B and C). Comparing gene expression before (0 h) and after (6 h) hESC dissociation, we found that the downregulation of TNF-related genes by Bcl-xL was independent of cell dissociation (Supplementary Fig. 6). These data demonstrated that Bcl-xL enhancing hESC survival may be mediated by increase of cell–cell adhesion and by decrease of death signaling.

Discussion

Unlike mouse ES cells that are capable of forming colonies from single cells, hESC growth depends on cell–cell interactions (Pyle et al., 2006). As a result, single-cell subculture of hESCs leads to few colonies due to cell dissociation-induced cell death. Currently, hESCs are propagated by mechanical dissection of hESC colonies into small clusters (Reubinoff et al., 2000; Klimanskaya et al., 2006) or mild collagenase dissociation into clusters of cells (Hasegawa et al., 2006; Sjogren-Jansson et al., 2005; Cowan et al., 2004; Suemori et al., 2006). Those subculturing methods have disadvantages in (i) large-scale expansion, (ii) uniform colony size controlling, (iii) seeding and differentiation with defined cell number, and (iv) single-cell required experiments.

To investigate apoptosis onset in hESC propagation, we explored the possibility of apoptosis attenuation and its effect on hESCs survival. In the established H1-Bcl-xL hESCs, an anti-apoptotic gene, Bcl-xL, is ectopically expressed by an inducible expression system. Our studies demonstrated that H1-Bcl-xL cells maintained the pluripotent markers and differentiation potential in vitro and in vivo. When H1-Bcl-xL hESCs was subcultured by the traditional method of mechanical scraping and collagenase treatment into cell clusters, the colony numbers, colony size, colony morphology, and gene expression of pluripotent markers were not affected by Bcl-xL overexpression, suggesting that hESC self-renewal capability is not affected by Bcl-xL expression. Importantly,

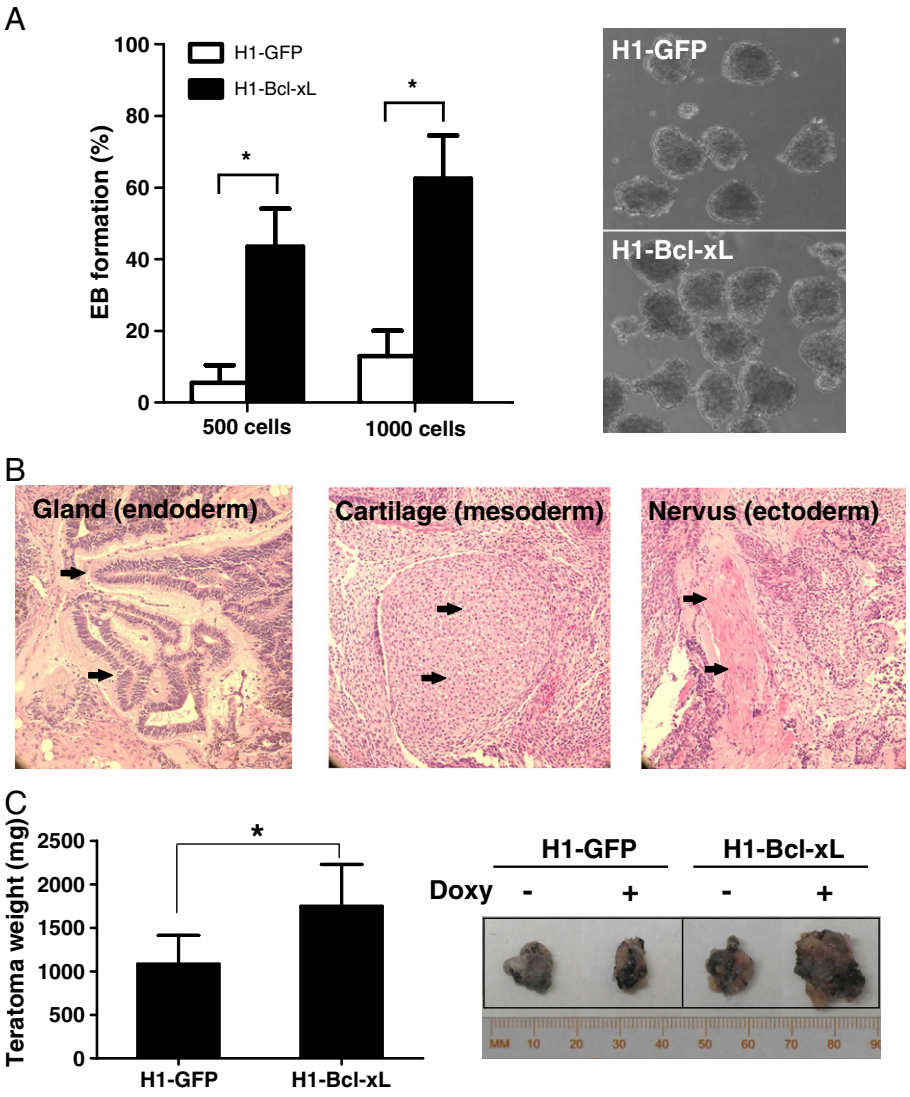

Figure 5 Increase of EB formation in vitro and teratomas in vivo by overexpression of Bcl-xL in hESCs. (A) Efficiency of EB formation and representative photos of EBs from H1-GFP control cells and H1-Bcl-xL cells. HESCs were dissociated into single cells by Accutase to form EBs (500 or 1000 cells per hanging drop) in differentiation medium with doxycycline (500 ng/ml) for 3 days. The percentages of EB formation were calculated based on the number of EBs from 100 drops in three independent experiments. (B) Teratomas formation in vivo of H1-Bcl-xL cells. Tissues of three germ layers in the teratomas from H1-Bcl-xL cells were analyzed after 8 weeks of cell implantation in nude mice. Photos were taken under 200× magnification. (C) Increase of teratoma sizes by Bcl-xL. Left panel: The weight of teratomas generated from H1-GFP and H1-Bcl-xL cells was determined after 8 weeks of injection with doxycycline. *$p<0.05$ is considered statistically significant. Right panel: Examples of teratomas generated from H1-GFP and H1-Bcl-xL cells with or without doxycycline administration.

overexpression of Bcl-xL significantly increased colony numbers when H1-Bcl-xL hESCs were subcultured with single-cell suspensions. Moreover, the efficiency of EB formation in hanging-drops from single-cell suspension was significantly increased in H1-Bcl-xL cells. Our studies suggest that large-scale expansion of hESCs from signal cells after dissociation can be achieved by attenuation of apoptosis. During our manuscript preparation, a report by Ardehali R, et al. (2011) showed that ectopic expression of Bcl-2 significantly decreased hESC dissociation-induced apoptosis (Ardehali et al., 2011). Therefore, attenuation of the apoptotic pathway by either overexpression of Bcl-xL or Bcl-2 enhances hESC survival.

Apoptosis can be initiated either by activation of death receptors on the cell surface membranes (extrinsic pathway) or through a series of cellular events primarily processed in the mitochondria (intrinsic pathway). Apoptosis involves cascades of caspases and Bcl-2 family members for its execution and regulation (Steller, 1995). The Bcl-2 family delivers strong impacts on pivotal decisions regarding cell survival regulation (Schendel et al., 1998). As an anti-apoptotic member of the Bcl-2 family, Bcl-xL targets mitochondrial apoptotic pathways (Kang and Reynolds, 2009; Chen et al., 2009). Overexpression of Bcl-xL improves cell survival against apoptotic signals induced by a variety of

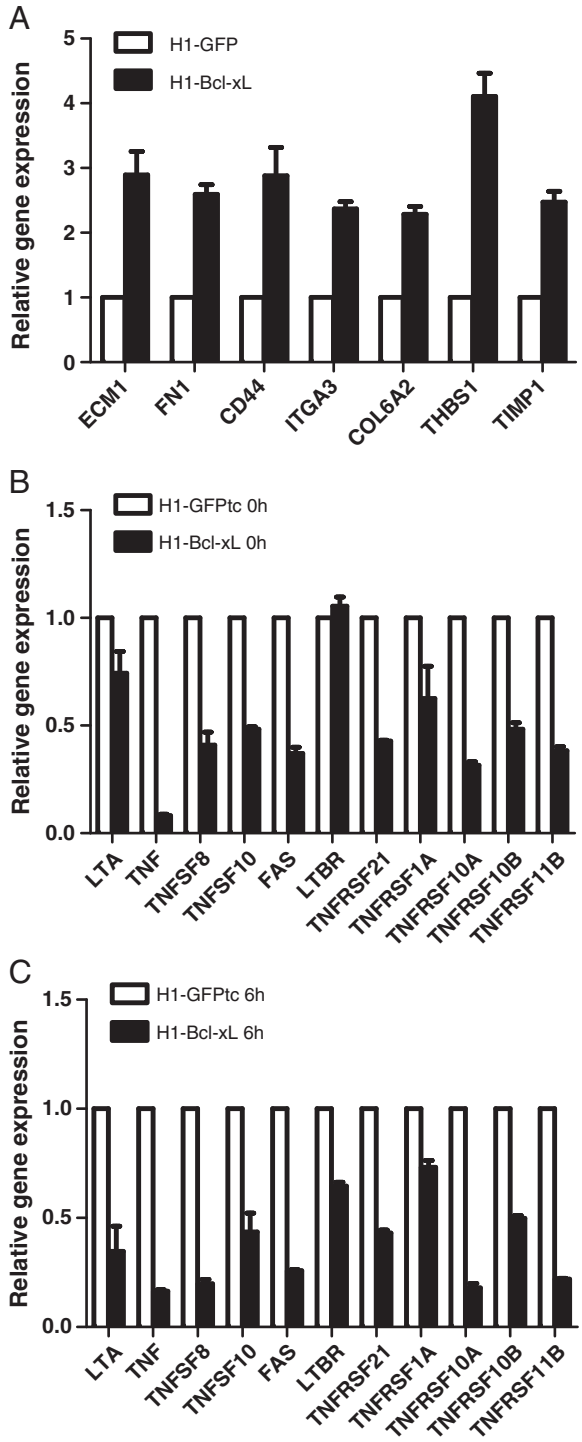

Figure 6 Regulations of adhesion molecules and TNF signaling mediators by Bcl-xL. (A) Gene expression analysis of adhesion molecules from H1-Bacl-xL cells and H1-GFP cells by qPCR array. HESCs were dissociated into single cells by Accutase and cultured on ultra-low attachment plates in presence of doxycycline (500 ng/ml). After 0 h (B) and 6 h (C) of cell dissociation, gene expressions of the TNF-related ligands and receptors were analyzed by qPCR array.

treatments including viral infection, UV and γ-radiation, heat shock, and agents that promote formation of free radicals (Kang and Reynolds, 2009; Chen et al., 2009). Apoptotic signals trigger the caspase cascade in part through Bcl-xL, and eventually activate caspase-3 to cleave death substrates (Swanton et al., 1999). In our study, the antibodies that specifically recognize the large subunit (approximate 20kD) of activated caspase-3 (Feng et al., 2003; Takeuchi et al., 2004) were used to evaluate apoptosis in hESCs. The number of caspase-3$^+$ cells quickly increased after trypsin or Accutase treatment aimed at single cell preparation from hESCs, indicating that disruption of cell–cell and cell–matrix interaction induced apoptosis. Indeed, the expression of many adhesion genes was elevated in H1-Bcl-xL hESCs. The upregulation of adhesion genes is independent of cell dissociation.

In addition, our gene expression analysis demonstrated that several TNF-related ligands and receptors were downregulated by overexpression of Bcl-xL in hESCs. A subgroup of the TNF-receptor superfamily is identified as death receptors with a predominant function in apoptosis induction (Schulze-Osthoff et al., 1998). TNF related-ligands bind to death receptors and induce receptor oligomerization, followed by the recruitment of an adaptor protein to the death domain through homophilic interaction. The adaptor protein then binds a proximal caspase, thereby connecting receptor signaling to the apoptotic effector machinery (Schulze-Osthoff et al., 1998). Our study demonstrated that the effect of Bcl-xL on hESC survival was executed through multiple pathways, including upregulation of adhesion molecular genes and downregulation of TNF-related death signals. How Bcl-xL regulates expression of adhesion and TNF-related molecules remains unknown.

Various cytokines and downstream signaling pathways, including FGF, BMP4 (Qi et al., 2004; Xu et al., 2005), TGFβ (Watabe and Miyazono, 2009; Peiffer et al., 2008), p38 MAPK (Binetruy et al., 2007), JNK pathway (Brill et al., 2009), and ERK pathway (Armstrong et al., 2006; Li et al., 2007) regulate hESC self-renewal. Growth factors also influence apoptosis via PKC, PI3K, and Akt pathways (Thompson and Thompson, 2004). Our study using inhibitors for specific signaling pathways indicated that Bcl-xL promoted single-cell survival of hESCs independent of those signaling pathways (Supplementary Fig. 7).

Improvement of hESC survival from single-cell culture should facilitate large-scale cultivation, and enable reliable differentiation and manipulation procedures of human pluripotent stem cells.

Materials and methods

Maintenance of hESC cultures

The H1 and H9 hESCs were obtained from WiCell Research Institute (Madison, WI). Human foreskin fibroblasts, Hs27 cells, (ATCC, Manassas, VA) were used as feeder cells to maintain the hESCs. The hESCs (passages 29–60) were grown on mitotic-inactivated Hs27 cells in hESC growth medium containing DMEM/F-12 (Invitrogen, Carlsbad, CA), 20% knockout serum replacement (KSR, Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Mediatech, Inc, Herndon, VA), 0.1 mM beta-mercaptoethanol (Sigma, St. Louis, MO), and 4 ng/ml FGF2 (R&D, Minneapolis, MN or PeproTech, Rocky Hill, NJ).

Hs27 cells were cultured in hESC growth medium without FGF2, and were used for up to 15 passages as hESC feeder cells. For hESC culture, Hs27 cells (2×10^4 cells per cm^2) were inactivated by mitomycin C (10 μg/ml) and seeded on 0.1% gelatin-coated plates. The hESCs were subcultured every 7 days by collagenase type IV treatment (1 mg/ml, Invitrogen) followed by mechanical scrapping. The hESC growth media were changed daily as previously described (Wang et al., 2007; Chen et al., 2007).

To remove feeder cells, hESCs were grown on Matrigel-coated plates in Hs27-conditioned media containing FGF2 (10 ng/ml). To induce ectopic expression of Bcl-xL, doxycycline (500 ng/ml, Sigma) was added into the growth medium 2 days before the experiments. To generate single-cell suspension, hESCs were treated with Accutase (Innovative Cell Technologies, CA) at 37 °C for 5 min. The cells were dissociated with gentle agitation. Single-cell suspensions were prepared by passing dissociated cells through a 30 μm cell strainer.

Generation of H1-Bcl-xL cells by lentiviral transduction

The human Bcl-xL gene was cloned into a lentiviral vector pLentiGFPtc, in which Bcl-xL expression was driven by a mini-CMV inducible promoter, and constitutive expression of fluorescence marker GFP was driven by an individual EF-1alpha promoter. The lentiviral vector pLentiGFPtc-Bcl-xL and control vector pLentiGFPtc, were transfected into 293T cells respectively for lentivirus preparation. The lentivirus was concentrated by PEG-8000 and applied to transduce the hESCs, as previously described (Wang et al., 2007). Using fluorescence microscopy, the GFP^+ hESC colonies were manually picked up. After five passages of selection, the hESCs capable of induced expression of Bcl-xL (H1-Bcl-xL) and the control cells (H1-GFP) were established.

Differentiation of hESCs

To induce differentiation of hESCs, undifferentiated hESCs were maintained on Matrigel-coated plates for 1 week to remove feeder cells, then treated with Dispase (1 mg/ml, Invitrogen) at 37 °C for 10 min to generate EBs, as previously described (Wang et al., 2007; Chen et al., 2007). EBs were formed with or without doxycycline (500 ng/ml, Sigma) in differentiation medium containing IMDM (Mediatech), 15% FBS (Hyclone, Logan, UT), 0.1 mM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Mediatech), and 450 μM monothioglycerol (MTG, Sigma). The differentiation medium was changed every 3 days. The differentiated hESCs were harvested at different time-points (3, 6, 9, 12, 15, 18, and 21 days) for analyses.

Western blot analysis

Expression of Bcl-xL was monitored by Western blot analysis. To induce Bcl-xL expression, doxycycline of various concentrations (0, 500, and 1000 ng/ml) was added to the hESC growth medium for 2 days, and then the cells were lysed in RIPA buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 μM leupeptin, 150 mM NaCl, 50 mM Tris, pH 7.4) supplemented with 1% protease inhibitor cocktail (Sigma). Western blot analyses were performed with anti-Bcl-xL antibodies (BD Biosciences) as primary antibodies, and anti-rabbit IgG-HRP antibodies (Sigma) as secondary antibodies. The protein expression levels were quantified using Photoshop software based on band area and gray scale.

RT-PCR and real-time quantitative PCR (qPCR) analyses

Total RNAs from undifferentiated hESCs or differentiated hESCs at different time points were isolated using Trizol (Invitrogen). To eliminate DNA contamination, the RNA samples were treated with DNase (Invitrogen) and cleaned by RNeasy kit (QIAGEN) before the reverse transcription (RT) reaction. Total RNA (~100 ng) was used for each reverse transcription reaction with SuperScript III (Invitrogen). qPCR was performed on iQ5 thermal cycler (Bio-Rad). Samples were adjusted to yield equal amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. Oligonucleotide primers and PCR conditions are listed in the Supplementary Table 1 (RT-PCR) and Table 2 (qPCR). The qPCR array analyses for adhesion molecules and apoptosis were performed by following the manufacturer's instructions (SABiosciences — QIAGEN, Frederick, MD).

Immunohistochemistry

For immunostaining, the cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min, permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min, and then incubated with 1% BSA for 30 min to block nonspecific binding. The cells were incubated for 1 h with the primary antibodies SSEA-4, TRA-1-60, and TRA-1-81 (all from Chemicon International Inc.), washed three times, and then incubated with rabbit anti-mouse Alexa594 antibodies (Invitrogen) for 1 h. The results were examined by a fluorescence microscope.

Flow cytometric analysis

HESCs were cultured on Matrigel-coated plates for 4 days, and treated with Accutase (Innovative Cell Technologies, CA) at 37 °C for 5 min. The cells were dissociated with gentle agitation. Single-cell suspensions were prepared by passing dissociated cells through a 30 μm cell strainer. Single hESCs (1×10^5 per well) were cultured on 24-well ultra-low attachment plates in hESC growth medium. Caspases are synthesized as precursors that undergo proteolytic maturation in apoptosis, either autocatalytically or in a cascade by enzymes with similar specificity. An active caspase consists of two large and two small subunits that form two heterodimers which associate in a tetramer. To examine the apoptosis, the APOACTIVE 3 kit (Cell Technology, Inc., Mountain View, CA), which is highly specific for the subunit of cleaved caspase-3 (activated caspase-3), was used to detect activated caspase-3. Briefly, the cells were harvested at different time points (0, 3, 6, 9, and 12 h), fixed by fixative solution, and then resuspended in PBS supplemented with 2% BSA to block nonspecific binding. The anti-caspase-3 antibodies and goat anti-rabbit IgG-phycoerythrin (PE) antibodies were used as primary and secondary antibodies respectively for flow cytometry. 7-Amino-Actinomycin D (7-AAD, BD Biosciences) was used to detect dead cells. Isotype-matched control

antibodies (BD Biosciences) were used to determine the background staining. The cells were analyzed on FACSCalibur (BD Biosciences) with CellQuest software. Data analysis was performed using CellQuest or FlowJo Software.

Generation of teratoma in nude mice

To remove feeder cells, undifferentiated hESCs were maintained on Matrigel-coated plates for a week. The hESCs were treated with Accutase to generate single-cell suspensions as described above. The cells (5×10^6) were mixed with Matrigel (5 mg/ml) in a final volume of 50 μl, and injected into the hindlimb of 8-week-old male NIH III nude mice. To induce Bcl-xL expression, the mice were fed doxycycline (200 μg/ml) containing drinking water starting 1 week before cell injection. The drinking water was changed every 3 days. The mice were sacrificed 8 weeks after the hESC injection to analyze the teratomas.

Teratomas were harvested, fixed for 24 h in 4% neutral buffered paraformaldehyde, transferred into 70% ethanol, and then examined by a routine wax-embedding histological procedure. Five-micrometer paraffin sections were mounted on slides and stained with hematoxylin and eosin.

Statistical analysis

The data were subjected to statistical analysis by the Student's *t*-test. Results with values of $p<0.05$ were considered statistically significant.

Author contributions

HB: collection and assembly of data, manuscript writing; KC, YXG, MA, YLX, and CM: collection and assembly of data; YGY and WSW: data analysis and interpretation; ZZW: conception and design, data analysis and interpretation, and manuscript writing.

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