

# Doxycycline Enhances Survival and Self-Renewal of Human Pluripotent Stem Cells

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<http://dx.doi.org/10.1016/j.stemcr.2014.06.013>

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## SUMMARY

We here report that doxycycline, an antibacterial agent, exerts dramatic effects on human embryonic stem and induced pluripotent stem cells (hESC/iPSCs) survival and self-renewal. The survival-promoting effect was also manifest in cultures of neural stem cells (NSCs) derived from hESC/iPSCs. These doxycycline effects are not associated with its antibacterial action, but mediated by direct activation of a PI3K-AKT intracellular signal. These findings indicate doxycycline as a useful supplement for stem cell cultures, facilitating their growth and maintenance.

## INTRODUCTION

Human embryonic stem and induced pluripotent stem cells (hESC/iPSCs) provide valuable platforms for developmental biology, disease modeling, and regenerative medicine (Ludwig et al., 2006; Reubinoff et al., 2000; Thomson et al., 1998; Yu and Thomson, 2008). Technically, however, hESC/iPSCs are difficult to culture, displaying slow growth and poor survival, especially upon cellular detachment and dissociation. Thus, hESCs were originally cultured in clusters on supporting feeder layers (Reubinoff et al., 2000). Feeder-free culture is possible if hESC/iPSCs are grown in Matrigel with chemically defined medium (Ludwig et al., 2006). In addition, methods such as culturing hESC/iPSCs in suspension (Steiner et al., 2010), with microcarriers (Bardy et al., 2013), or on synthetic polymers (Villa-Diaz et al., 2013) have been introduced. These new techniques, however, are expensive, have limited scalability, and may have high batch-to-batch variability. Y-27632, a ROCK inhibitor, is used to prevent cell apoptosis after cell dissociation and to promote cell viability after plating (Ohgushi et al., 2010; Watanabe et al., 2007), but the benefit of this chemical is limited to a brief period after cell dissociation and its continued effects on cell survival and proliferation are questionable (Couture, 2010). Thus, culture methods that are low cost, robust, scalable, easy to use, and consistent remain to be further developed to allow widespread applications of hESC/iPSCs in basic research and clinical.

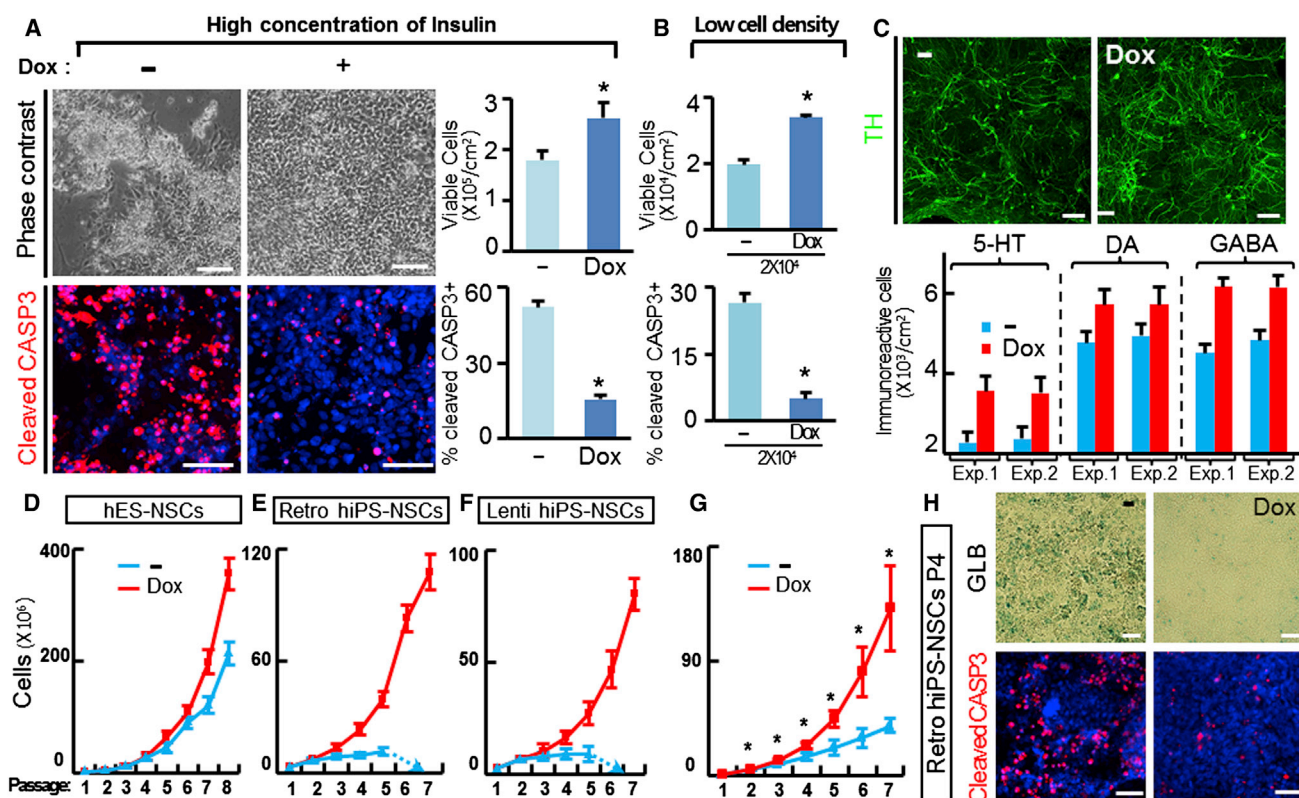
Human neural stem cells (hNSCs) were derived by *in vitro* differentiation of hESC/iPSCs. The hESC/iPSC-derived NSCs (hES/iPS-NSCs) homogeneously expressed

nestin, a representative NSC-specific marker (Park et al., 2005). These nestin<sup>+</sup> hNSCs can be expanded with basic fibroblast growth factor (bFGF) and differentiate toward neuronal cells upon withdrawal of bFGF and in the presence of combinatory neurotrophic factors (Park et al., 2005; Perrier et al., 2004). In an experiment involving a doxycycline-inducible expression system, we accidentally observed that doxycycline (1  $\mu$ g/ml) itself promoted hNSC survival; the cell survival effect was not associated with exogene expression. These observations prompted us to scrutinize the doxycycline action further in undifferentiated hESC/iPSC cultures.

We herein show that simple supplementation with doxycycline greatly improves both hESC/iPSC viability and self-renewal. In practice, doxycycline dramatically enhances hESC/iPSC expandability, and the effects continue for long time periods. The effects of doxycycline are mediated by direct activation of the PI3K-AKT intracellular pathway, which has recently been reported as the most crucial signal for hESC/iPSC self-renewal (Bendall et al., 2007; Singh et al., 2012).

## RESULTS

We recently showed that hNSCs are extremely sensitive to insulin, an indispensable culture supplement, and thus survival of these cells is ensured only within a narrow range of low insulin concentrations (Rhee et al., 2013). In addition, survival of hNSC derived from hESC/iPSCs is greatly dependent on the cultured cell density.



**Figure 1. Effects of Doxycycline Supplementation on Cultures of hNSCs and Neurons Derived from hESC/iPSCs**

(A and B) Doxycycline prevents apoptotic cell death induced by high concentrations of insulin (A) or low cell density (B) in hNSC cultures. H9 hESC-derived NSCs plated at  $1.4 \times 10^5 / \text{cm}^2$  (A) or  $2 \times 10^4 / \text{cm}^2$  (B) were cultured with or without doxycycline supplementation (1  $\mu\text{g}/\text{ml}$ ). Apoptotic cell deaths were estimated by viable cell counts (upper) and cells positive for activated (cleaved) caspase 3 (lower). \*Significantly different from untreated control at  $p < 0.01$ , Student's t test,  $n = 3$  replicates in duplicate. Representative phase-contrast and cleaved caspase-3-stained images in the cultures with the high insulin concentration (5  $\mu\text{M}$ ) are shown in the left panels. Scale bars, 50  $\mu\text{m}$ . (C) The effect of doxycycline on neuron survival. Subtypes of neurons were derived by differentiation of H9 hES-NSCs for 9 days. The neuronal cultures were maintained with or without doxycycline supplementation for 5 days.  $n = 4$  technical replicates/experiment,  $n = 2$  experiments. On the last day of culture, immunostaining was performed to identify dopaminergic (tyrosine hydroxylase, TH), serotonergic (serotonin), and GABAergic (GABA) neurons. Scale bars, 50  $\mu\text{m}$ .

(D–H) Doxycycline supplementation prevents early senescence and apoptotic cell death in viral hiPS-derived NSCs during cell passaging. NSCs derived from the retroviral (Rv-hiPS 02-3, E), lentiviral hiPSC lines (IMR 90-1, F), and H9 hESCs (control, D) were expanded by passaging every 7 days in the absence or presence of doxycycline supplementation. The cell growth curves shown in (D)–(F) were generated by counting total viable cell numbers from  $n = 3$  technical replicates of each doxycycline-treated and untreated culture on the last day of every passage. (G) Statistical analysis on the cell number increases from the three cell lines tested was carried out ( $n = 3$  biological replicates). \*Significant increases of viable cell numbers in the doxycycline-supplemented cultures at  $p < 0.001$ , Student's t test. (H) Representative  $\beta$ -galactosidase (upper, cellular senescence) and activated caspase-3 (lower, apoptosis)-stained cells in the Retro-2-hiPS-NSC cultures untreated (left) and treated with doxycycline (right) on the last day of passage 4. Scale bars, 50  $\mu\text{m}$ . All data shown in this study are expressed as mean  $\pm$  SEM.

See also Figure S1.

Doxycycline supplementation (1  $\mu\text{g}/\text{ml}$ ) strikingly prevented apoptotic cell death induced by high insulin concentration or low cell density in H9 hESC-derived NSC cultures, as revealed by estimations of total numbers of viable cells and cells positive for activated caspase-3 (Figures 1A and 1B) and Annexin V/propidium iodide (PI) (data not shown). We then tested the effects of doxycycline

on differentiated neurons. Dopamine, serotonin, and gamma-aminobutyric acid (GABA)-secreting neurons are derived by terminal differentiation of hES/iPS-NSCs. The neurons underwent apoptosis if the neurotrophic factors supplemented were withdrawn, which substantially reduced the numbers of each neuronal subtype. Doxycycline largely prevented such apoptosis and neuronal loss



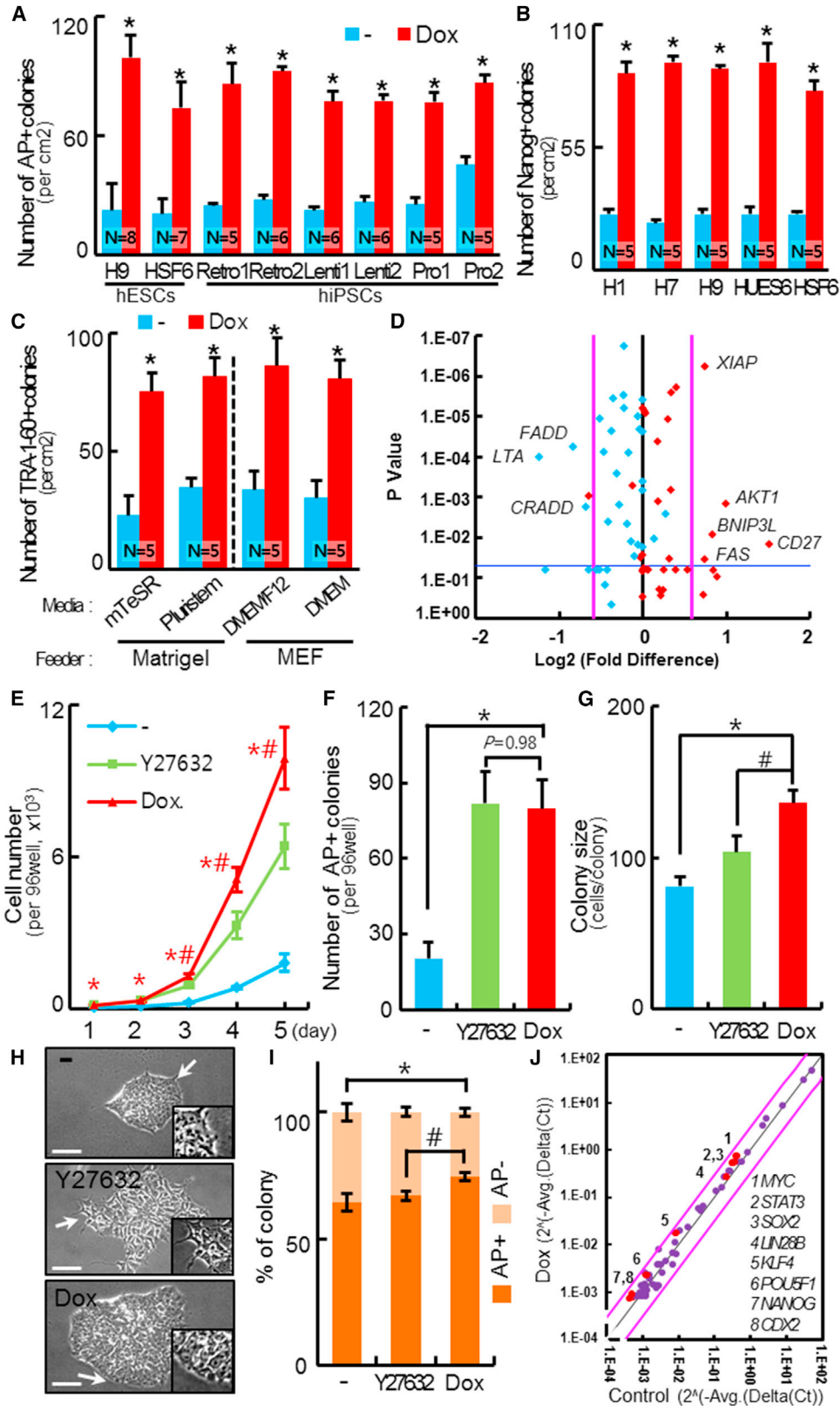
(Figure 1C). Doxycycline from different sources (companies) showed similar effects on hNSC survival (Figures S1A and S1B available online). The doxycycline analogs tetracycline and minocycline also promoted cell survival, but other antibacterial agents did not, indicating that doxycycline-mediated cell survival is not associated with its antibiotic effect.

NSCs derived from hESCs (hES-NSCs) are highly expandable for prolonged periods of proliferation through multiple cell passages (Hong et al., 2008; Ko et al., 2007). Doxycycline increased cell yields attained during expansion of H9 hES-NSCs through eight passages (Figure 1D). Neuronal differentiation of the multipassaged hNSCs treated with doxycycline was not altered (data not shown). In contrast to hES-NSCs, we have recently shown that NSCs derived from hiPSCs established using viral vector-mediated gene delivery (viral hiPSCs) exhibited early senescence and apoptotic cell death during passaging (Rhee et al., 2011). Thus, cultures of NSCs derived from viral hiPSCs (viral hiPS-NSCs) were barely maintained for two to four cell passages; no definite cell expansion took place and at the end of this period, none were viable (Rhee et al., 2011) (Figures 1E and 1F). Hemangioblasts derived from viral hiPSCs versus hESCs likewise exhibited differences in expansion potential and senescence (Feng et al., 2010). Doxycycline supplementation strikingly prevented senescence of retroviral- and lentiviral-based hiPS-NSCs during passaging (Figure 1H; data not shown). Indeed, doxycycline addition allowed the viral hiPS-NSC cultures to be maintained for at least seven cell passages and to undergo discrete cell number increases (Figures 1E and 1F). These findings strongly indicate that doxycycline supplementation circumvents the limitation of viral hiPS-NSCs as a stable cell source.

Based on doxycycline-mediated effects on cell survival in neural lineages at different developmental stages, we postulated that the effects of doxycycline and its analogs would extend to cells at earlier developmental stages, such as pluripotent stem cells. Culturing human pluripotent hESC/hiPSCs is technically difficult, with problems in maintaining self-renewal and pluripotency for long periods of culture and massive cell death occurring, particularly after cell dissociation. To examine the effect of doxycycline on human pluripotent stem cell survival, hESC (H9) clusters were dissociated into one to five cells and plated on Matrigel in mTeSR1 medium in the presence or absence of doxycycline (1  $\mu$ g/ml). Doxycycline supplementation strikingly increased formation of colonies that were positive for alkaline phosphatase (AP), an undifferentiated hES/hiPSC marker, 5 days after plating (22.5  $\pm$  13.5 versus 99.3  $\pm$  11.2 colonies/cm<sup>2</sup> without versus with doxycycline, respectively). Enhanced AP<sup>+</sup> colony formation in the presence of doxycycline was also apparent in all other

hESC (HSF6) and hiPSC lines tested (Rv-hiPS 01-1 and Rv-hiPS 02-3 (retroviral-hiPSCs); IMR90-1 and IMR90-4 (lentiviral-hiPSC); piPS-#1 and piPS-#2 (protein based-hiPSC)) (Figure 2A). The doxycycline effect was further confirmed by counting colonies expressing the other pluripotent cell markers NANOG and TRA-1-60 in cultures of the commonly used hESC lines H1, H7, H9, HUES6, and HSF6 (Figure 2B; data not shown) and in different culture conditions (Figure 2C). In gene expression arrays, expression of 35 of 39 apoptosis-related genes was lower in doxycycline-treated versus untreated hESCs, whereas the opposite pattern occurred for antiapoptotic gene expression (Figure 2D).

Y-27632, a selective Rho-associated kinase (ROCK) inhibitor, is known to facilitate hESC/hiPSC culture growth by diminishing dissociation-induced apoptosis (Ohgushi et al., 2010; Watanabe et al., 2007). We therefore compared doxycycline- and Y-27632-mediated hESC survival by the colony-forming assay using H9 cells in Matrigel coating-mTeSR1 medium culture. Cell numbers increased more during 5 days of culture in the doxycycline-supplemented cultures than in either the untreated or Y-27632-treated cultures (Figure 2E). The number of colonies (colony-forming efficiency) was robustly greater in the cultures treated with doxycycline (81.9  $\pm$  12.7 colonies/well) or Y-27632 (79.6  $\pm$  11.7 colonies/well) than in the untreated control (20.3  $\pm$  6.7 colonies/well); the doxycycline and Y-27632 effects were indistinguishable (Figure 2F). However, colony sizes (average numbers of cells assembled in a colony) in the doxycycline-treated group (136.2  $\pm$  8.1 cells/colony) were significantly larger than those in the Y-27632-treated group (103.8  $\pm$  10.5 cells/colony) and untreated control (80.9  $\pm$  6.3 cells/colony) (Figure 2G). Cell colonies in Y-27632-treated cultures differed somewhat in shape as compared with those of the control cultures, with less compactly arranged cells that exhibited bipolar cellular processes (Figure 2H), probably because of altered Rho kinase activity, which is associated with changes in cell morphology (Harb et al., 2008) (the cell morphologies returned to normal within several hours after Y-27632 withdrawal). In contrast, cell and colony shapes in the doxycycline-treated cultures were indistinguishable from those of the untreated culture. Notably, the percent of AP<sup>+</sup> undifferentiated hESC colonies in the total number of colonies was significantly higher in the doxycycline-treated cultures than in the untreated control and Y-27632-treated cultures (74.7%  $\pm$  1.6% in doxycycline-treated, 66.9%  $\pm$  1.8% in Y-27632-treated, 64.3%  $\pm$  3.3% in untreated cultures, Figure 2I). Expression of genes encoding human transcription factors generally appeared similar for doxycycline-treated and untreated hESCs in gene expression arrays, but expression levels of genes related to hESC pluripotency were greater in the doxycycline-treated cultures (Figure 2J).



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The increased pluripotency marker gene expression was confirmed using real-time PCR (data not shown). Doxycycline's ability to maintain an undifferentiated state was also apparent in mouse ESCs (mESCs) that had been engineered to contain *Oct3/4* promoter-driven GFP: doxycycline treatments increased the percentage of GFP<sup>+</sup> cells (Figure S2). Taken together, these findings indicate that doxycycline supplementation generates greater yields of undifferentiated hESCs in culture, not only by promoting cell survival but also by maintaining self-renewal.

Because of their vulnerability after cell dissociation, hESC/iPSCs are commonly maintained in clusters without dissociation on a feeder layer of mouse embryonic fibroblasts (MEFs). Thus, we examined whether doxycycline supplementation would confer a practical benefit: facilitating the expansion and maintenance of undifferentiated hESC/iPSCs in culture for long periods with multiple cell passages. The doxycycline-mediated hESC survival and self-renewal shown in colony-forming assays (Figure 2) were further confirmed using fluorescence-activated cell sorting (FACS) on hESC/iPSCs cultured in clusters (Figures 3A and 3B). Cells on apoptosis (Annexin V<sup>+</sup>/PI<sup>+</sup>) were greatly reduced by doxycycline treatment (Figure 3A), along with decrease of dead cell populations (Figure 3B). In addition, significantly more cell populations in hESC (H9) and hiPSC (Lenti-1) cultures were accumulated in S phase by doxycycline treatment (Figure 3B). When H9 hESCs were subcultured in clusters on MEF feeders, increases of >5-fold and >120-fold in cell num-

ber were attained over 56 days (8 passages) and 140 days of culture (20 passages), respectively, by simply adding doxycycline (Figures 3C and 3D), as compared with untreated cells, without altering the normal karyotype (Figure 3E). Immunocytochemical (Figure 3F) and FACS (Figure 3G) analyses showed that, in the multipassaged cultures with doxycycline supplementation, most cells expressed undifferentiated hESC markers. In addition, pluripotent potentials of the hESCs maintained with doxycycline supplementation were confirmed by in vitro differentiation into three germ layers (Figures S3A and S3B) and in vivo teratoma formation (Figure S3C).

Y-27632 has been reported to permit survival of dissociated hESCs by inhibiting actomyosin hyperactivation, which is triggered by the loss of E-cadherin (CDH1)-dependent intercellular contact (Ohgushi et al., 2010). In control cultures, loss of E-cadherin was obvious after cell dissociation and followed by increased levels of phosphorylated myosin light chain (pMLC), a marker for actomyosin hyperactivation, within 30 min after hESC dissociation (Figure 4A). As reported, pretreatment with Y-27632 substantially prevented the loss of E-cadherin and actomyosin hyperactivation. In contrast, the cell dissociation-induced changes were not prevented by doxycycline pretreatment (Figures 4A and 4B). Consequently, atypical blebbing, a characteristic feature of hyperactivated actomyosin, occurred in doxycycline-pretreated cultures (Figure 4C), as it does in untreated hESCs after

### Figure 2. Doxycycline Supplementation Enhances Cell Survival and Self-Renewal of Undifferentiated hESC/iPSCs in Culture

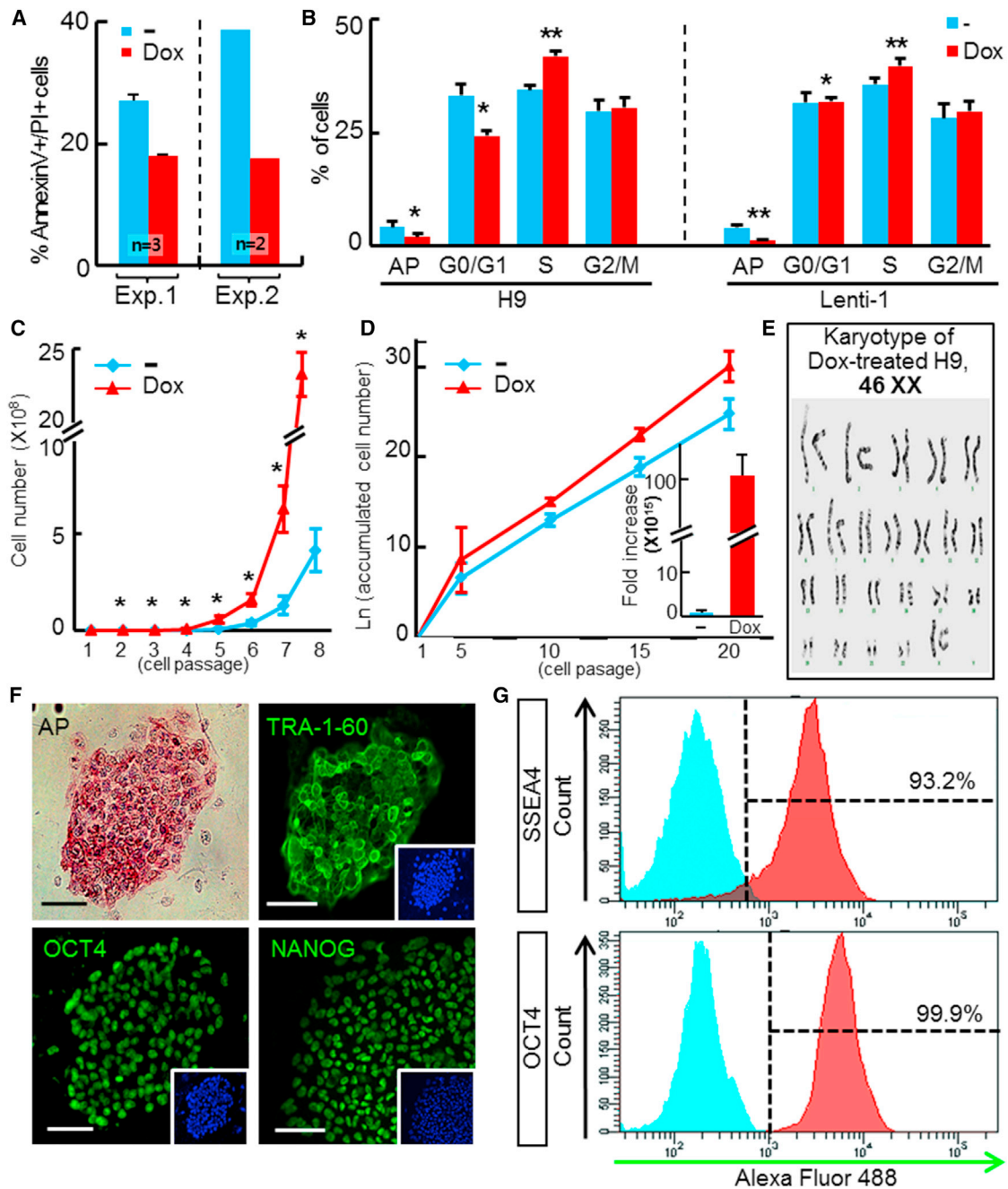
(A–C) Colony-forming efficiency. hESC/iPSCs were dissociated into one to five cells, and plated at  $4 \times 10^4$  cells/6 cm dish in feeder-free Matrigel with mTeSR1 medium in the absence or presence of doxycycline (1  $\mu$ g/ml). After 5 days, colonies expressing the undifferentiated hESC/iPSC markers AP (A), NANOG (B), or TRA-1-60 (C) were counted. Information for the cell lines used is shown in Table S1. \* $p < 0.01$ (A), \* $p < 0.05$ (B), Student's two-tailed t test.  $n = 5\sim 8$  (A and B) pairs of doxycycline-treated and untreated culture dishes for each cell line. Numbers of experiments for each assay are indicated on the graphs. Data within pairs were collected at the same time, but across pairs were collected at different times and presented in one graph for ease. The effect of doxycycline on TRA-1-60<sup>+</sup> colony formation was also assessed in the different culture conditions indicated using the H9 hESC line (C). \* $p < 0.01$ ,  $n = 5$  experiments for each group.

(D) Expression arrays for anti-/proapoptotic genes altered by doxycycline treatment. H9 hESCs were cultured in the absence or presence of doxycycline and expression levels of anti-/proapoptotic genes were estimated using RT<sup>2</sup> PCR Profiler Array. Shown is a "volcano plot" demonstrating a tendency of increased antiapoptotic (red dots) and decreased proapoptotic (blue dots) transcripts in doxycycline-treated cultures (technical triplicates). Each dot is mean value of the triplicates. Vertical pink lines indicate the 1.5-fold change in gene expression threshold. The gene names significantly up- or downregulated by >1.5-fold at  $p < 0.05$  (horizontal blue line) by doxycycline treatment are shown in the graph.

(E–I) Comparison of the effects of doxycycline and Y-27632 on hESC survival and proliferation. Dissociated H9 cells were plated at a low cell density (500 cells/well, 96-well plate) and cultured on Matrigel in mTeSR1 without or with supplementation of doxycycline (1  $\mu$ g/ml) or Y-27632 (10  $\mu$ M) for 5 days. Cell growth for 5 days is shown in (E). Numbers (F) and sizes (G) of the colonies formed were compared. \* $p < 0.001$  compared to the control group, # $p < 0.05$  compared to Y-27632-treated group, One-way ANOVA with Tukey post hoc analysis,  $n = 5$  experiments in triplicate. (H) Phase-contrast images of representative colonies from untreated, Y-27632-treated, and doxycycline-treated cultures. Insets, high-powered images of the indicated cells. Scale bars, 50  $\mu$ m. (I) % AP<sup>+</sup> undifferentiated colonies. \* $p < 0.01$  compared to the control group, # $p < 0.05$  compared to Y-27632-treated group,  $n = 9$  experiments, ANOVA.

(J) Expression levels of genes encoding human stem cell transcriptional factors in H9 cells treated with doxycycline were compared with expression levels in the untreated culture (technical triplicates). In the scattered plot, the pink lines indicate the 2-fold change in gene expression threshold. Genes whose expression is associated with pluripotency (red dots) are listed in the graph.

See also Figure S2.



**Figure 3. Practical Benefits of Doxycycline Supplementation in hESC/iPSC Cultures**

The effects of doxycycline supplementation were examined in undifferentiated hESC/iPSC cultures maintained in clusters without dissociation on MEF feeder cells.

(A and B) FACS analyses for determining the fraction of cells undergoing apoptosis (A) and the fraction that had accumulated in each cell-cycle phase (B). Small clusters of H9 hESCs or Lenti-1 hiPSCs (10-50 cells) were plated and cultured in the presence or absence of doxycycline (1 μg/ml) for 6 days. The hESC/iPSC clusters were mechanically harvested, dissociated using TrypLE (A) or Accutase (B), 2 hr prior to Annexin V/PI (A) or PI (B) staining. The stained populations were analyzed using FACS. n = 2 or 3 technical replicates/experiment, n = 2 experiments (A). n = 3 experiments (B) \*p < 0.05 and \*\*p < 0.01, paired t test. Populations of dead cells (AP, apoptotic population) in (B) were analyzed using the result of the sub-G1 area gated with PI.

(C-G) The effects of doxycycline treatment on the growth and maintenance of undifferentiated hESCs for long-term expansion. H9 hESCs were propagated in clusters on MEF feeders in bFGF-supplemented KSR medium with or without doxycycline with multiple passages. (C-D)

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dissociation (data not shown). Interestingly, activated (phosphorylated) AKT (pAKT) levels also decreased after hESC dissociation. Notably, pAKT levels remained stable if cultures were pretreated with doxycycline (Figure 4A). To determine which intracellular signaling proteins are responsible for the doxycycline-mediated survival and maintenance of undifferentiated hESCs, we initially screened the activities of 46 intracellular signaling proteins using human phospho-kinase arrays (Proteome Profiler, R&D Systems) (Figure 4D). Levels of phosphorylated (activated) ERK (pERK), pCREB, and  $\beta$ -catenin (CTNNB) were significantly greater in hESC cultures treated with doxycycline versus untreated, but the changes were slight and not substantiated in cytometric bead arrays (CBAs), another kinase immunoassay system (Figure S4). In contrast, activation of AKT (pAKT) in doxycycline-treated cultures was the most prominent change in the array and was further confirmed by western blot analyses of cells under different conditions, such as dissociated hESCs (Figure 4A) and cells plated in clusters (data not shown). The effect of doxycycline on pAKT attracted our interest because phosphoinositide 3-kinase (PI3K)-AKT intracellular signaling, in addition to its commonly known antiapoptotic role, has been revealed as the most crucial signaling pathway for the maintenance of hESC pluripotency and self-renewal (Bendall et al., 2007; Singh et al., 2012). We further observed that the doxycycline effects on hESC survival (Figure 4E), proliferation (Figure 4F), and maintenance of an undifferentiated state (Figure 4G) were reduced or eliminated by the PI3K inhibitors LY29007 and wortmannin. Notably, a conventional autoradiogram-based assay revealed that PI3K enzyme activity was obviously increased in hESC cultures supplemented with doxycycline (Figure 4H). We further carried out another type of PI3K activity/inhibitor assay, in which doxycycline and its analogs, but not other antibiotics tested, enhanced the PI3K activity in a dose-dependent manner (Figure 4I). Because the latter PI3Kase assay assesses enzymatic activity in a test tube containing the enzyme and substrate in the absence of cellular extracts or components, the observed doxycycline action must occur through direct action on the PI3K enzyme, rather than indirectly through other cellular molecules. Mechanisms for how doxycycline acts as a direct PI3K activator remains to be identified.

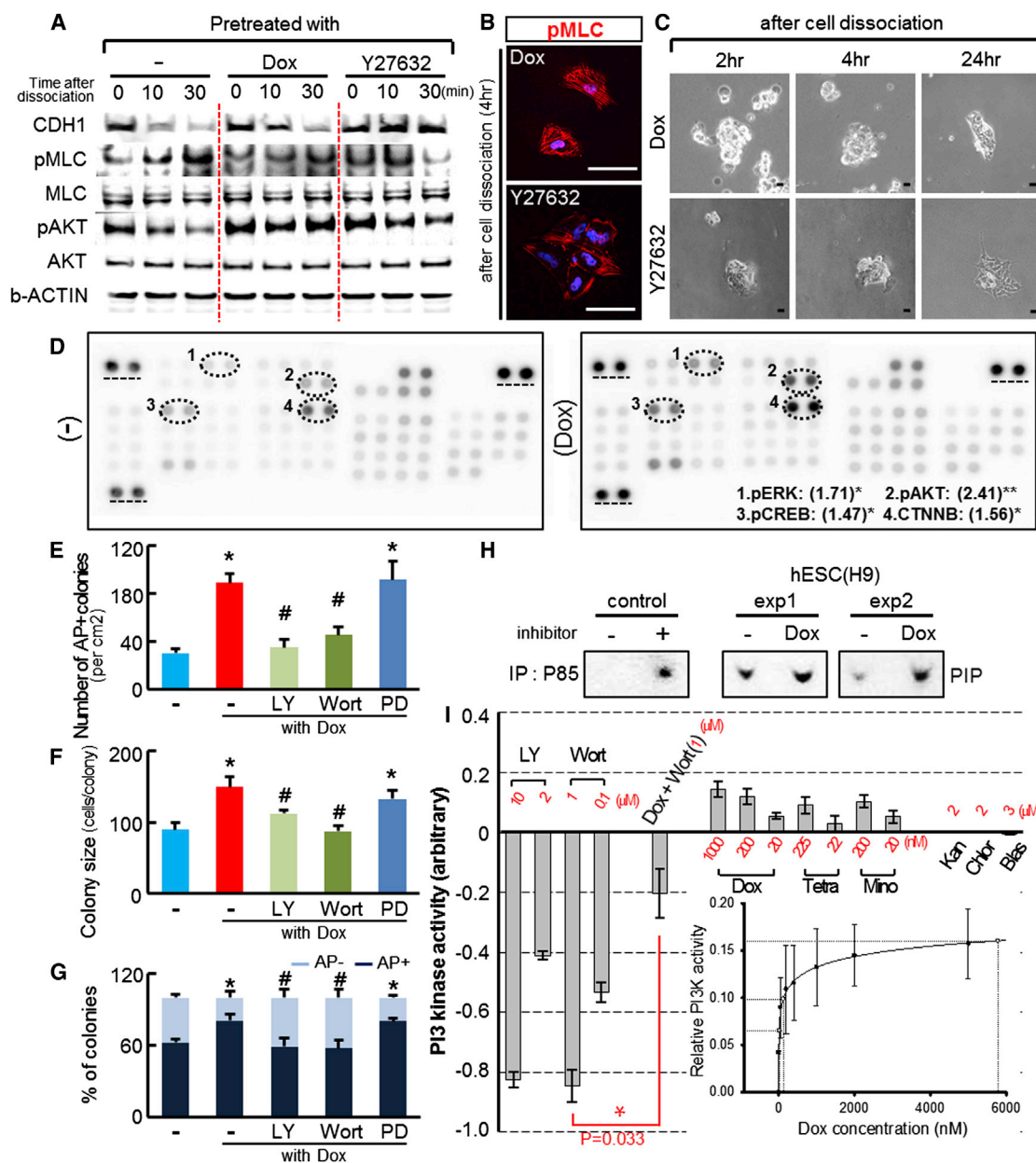
## DISCUSSION

This study began with the serendipitous observation that doxycycline treatment strongly enhances hNSC and neuron viability. Similar to our results, previous studies have shown that doxycycline and its analog minocycline are cytoprotective toward terminally differentiated neuronal cells (Jantzie et al., 2005; Yrjänheikki et al., 1998); as such, these drugs have been used to treat ischemic brain injury (Kim and Suh, 2009). Furthermore, a recent study has demonstrated that preconditioning rat brain-derived NSCs with minocycline increases the viability of the grafted NSCs after transplantation in rat stroke model (Sakata et al., 2012). The neuroprotective effects were reported to be attained by inhibition of microglial activation (Wang et al., 2005; Yrjänheikki et al., 1998), other anti-inflammatory actions (Hu et al., 2010), or inhibition of metalloproteinases (Garrido-Mesa et al., 2013). Given these mechanisms of action, doxycycline and its analogs have also been tested and found to enhance mesenchymal stem cell-driven cartilage repair and chondrogenesis (Lee et al., 2013). Although these extra-antimicrobial actions have been published for a number of years, the commonly used doxycycline-inducible gene expression system is often employed without regard for such activity. Our study suggests that this activity should indeed be considered when using this inducible expression system.

Our study extended the doxycycline-mediated cell survival effect to human pluripotent ESC/iPSCs. In addition, we show that hESC/iPSC self-renewal is promoted by doxycycline supplementation. Because microglia and inflammatory cells are not present in hESC/iPSC cultures and the doxycycline effects were manifested in isolated single cells, the effects must not be attained through doxycycline's reported extrinsic/environmental modulations. Instead, we show that in hESC/iPSCs, doxycycline directly activates the PI3K-AKT pathway, which has, in addition to its involvement in general cell survival, been reported to be the most crucial regulatory pathway for self-renewal of undifferentiated human pluripotent stem cells (Bendall et al., 2007; Singh et al., 2012). It is intriguing to consider how doxycycline might activate PI3K enzyme activity. However, it is difficult to predict the underlying mechanisms,

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Cell growth curves. The curves shown in (C) were generated by counting total viable cell numbers on the last day of every passage during eight passages for 56 days from five independent experiments. \*Significantly different from the respective untreated controls at the same cell passages at  $p < 0.001$ , Student's two-tailed  $t$  test. Doxycycline supplementation effect on cell growth was further examined up to 20 passages (for 140 days) from three technical replicate ( $n = 1$  experiment) (D). The curves shown in (D) are the natural logarithm (Ln) of accumulated total cell numbers and fold increases at passage 20 compared to passage 1 (inset). (E) Unaltered karyotype of H9 cells after 20 passages of doxycycline-supplemented cultures. The chromosomal analysis was done in 20 randomly chosen nuclei. (F and G) Immunocytochemical (F) and FACS (G) analyses showing the expression of undifferentiated hESC markers in the multipassaged H9 cell in the presence of doxycycline. Blue populations in (G) are negative controls without the primary antibody reactions. Scale bars, 50  $\mu\text{m}$ . See also Figure S3.



**Figure 4. Doxycycline Permits Cell Survival and Maintenance of Undifferentiated hESCs through PI3K-AKT Signal Activation.**

(A–C) Cell dissociation-induced actomyosin hyperactivation is prevented by Y-27632, but not by doxycycline treatment. H9 hESCs were incubated with doxycycline or Y-27632 or without these chemicals (control) for 1 hr prior to cell dissociation. Cells were then harvested 0, 10, and 30 min after cell dissociation and subjected to western blot analyses to detect proteins involved in cell-dissociation-induced apoptosis (A). Shown in (B) and (C) are representative pMLC-immunostained and phase-contrast images of dissociated hESCs in the cultures treated with Y-27632 or doxycycline. Cells pretreated with Y-27632 or doxycycline were dissociated, directly plated onto Matrigel with mTeSR1 and cultured in the continuous presence of the chemicals. The images were captured at the indicated time after plating (dissociation). Scale bars, 30  $\mu$ m.

(D) Estimation of intracellular signals activated by doxycycline. H9 hESCs were incubated without (control) or with doxycycline (1  $\mu$ g/ml) for 30 min before harvesting and then subjected to immunoblot analyses using the human phosphokinase blot array, which is designed to detect 46 phosphorylated intracellular proteins. The array analyses were performed in technical triplicate. Shown is a representative pair of blots of the untreated control and doxycycline-treated cells. In the blots, spots whose intensities are greater (>1.5-fold) in the doxycycline-treated group, compared to the control, are marked with dotted circles and listed with the fold increase (parentheses) in the inset. \*\* $p < 0.001$  and \* $p < 0.05$ , paired t test. Reference spots are underlined.

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such as which structural moieties of doxycycline might be responsible for its activator function; unlike enzyme inhibitors, intracellular kinase activators are uncommon and structure-based predictions for activator mechanisms are not feasible. Thus, the mechanism for doxycycline's effect on PI3K activity remains to be identified in future studies.

We show that hESC dissociation not only leads to actomyosin hyperactivation, but also reduces Akt signaling, events that are prevented by Y-27632 and doxycycline, respectively. Thus, these chemicals additively or synergistically prevented cell apoptosis for 1 day after cells were plated following cell detachment and dissociation (data not shown). However, unlike doxycycline, the long-term effects of Y-27632 are unclear and Y-27632 is associated with aneuploidy (Couture, 2010; Riento and Ridley, 2003) and cell morphology changes (Figure 2H). Therefore, the best hESC/iPSC growth was attained by combined treatment of doxycycline and Y-27632 for 1 day after plating, followed by doxycycline supplementation alone for the remaining culture period (data not shown).

In summary, our findings demonstrate that doxycycline promotes hESC/iPSC survival and self-renewal via PI3K-AKT signal activation. These effects are also apparent in NSCs and differentiated neurons; AKT activation was also observed in the cells of neural lineages (data not shown). Given that AKT activation is a common cell survival signal in many kinds of cells, these findings suggest that doxycycline can be used as a cell culture supplement in wide range of cellular types. Doxycycline and its analog minocycline are already being used in clinics, suggesting that doxycycline supplementation would be acceptable in the stem cell cultures used for clinical applications.

## EXPERIMENTAL PROCEDURES

### Cell Culture

hESCs and hiPSCs were grown based on hESC research guidelines approved by the institutional review board (IRB) at Hanyang University (Seoul, Korea) under HYE-08-02. hiPSCs and hESCs used in this study are listed in Table S1. For expansion and maintenance of

undifferentiated hESC/iPSCs, cells were cultured on a feeder layer of MEF cells (CF1 mouse, Charles River Kingston) in KSR medium (knockout serum replacements; Invitrogen) supplemented with 4 ng/ml basic fibroblast growth factor (bFGF, R&D Systems). The cultures were passaged every week in clusters by mechanically splitting (or by mild pipetting with collagenase IV [0.1 mg/ml; STEMCELL Technologies]) and transferring hESC/iPSC clusters onto freshly prepared MEF feeder layers. For colony-forming assays, hESC/iPSC clusters were dissociated into one to five cells using collagenase IV (0.1 mg/ml; STEMCELL Technologies) or Accutase (STEMCELL Technologies), plated at  $4 \times 10^4$  cells/6 cm culture dish (or 500 cells/well, 96-well plate) on Matrigel (BD Biosciences) with mTeSR1 (STEMCELL Technologies), without feeder layers. The colony-forming assay was also performed in different media conditions such as Pluristem (Millipore) with Matrigel, DMEM/F12-, or DMEM-based KSR medium on MEF feeder layers. KSR medium supplemented with bFGF and mTeSR1 medium was changed every day without special mention. hNSC cultures were derived by in vitro differentiation of hESCs and hiPSCs as previously described (Ko et al., 2007; Rhee et al., 2011). The hNSCs were expanded with bFGF (20 ng/ml) in ITSA media (Park et al., 2005; Rhee et al., 2011) and subcultured every week for long-term expansion. Terminal differentiation of the hNSCs was induced in the absence of bFGF but in the presence of brain-derived neurotrophic factor (20 ng/ml; R&D Systems), glial cell line derived neurotrophic factor (20 ng/ml; R&D Systems), or dibutyryl cAMP (0.5 mmol/l; Sigma). The following chemicals were used: doxycycline (Sigma; Calbiochem; Tocris), minocycline (Sigma), tetracycline (Sigma), kanamycin (Sigma), blasticidin (Invitrogen), chloramphenicol (Sigma), Y-27632 (Calbiochem), LY 294002 (Calbiochem), wortmannin (Millipore), and PD 98059 (Calbiochem).

### Immunostaining

Cultured cells were fixed with 4% paraformaldehyde in PBS and incubated overnight at 4°C with primary antibodies listed in Table S2. Appropriate fluorescence-tagged secondary antibodies (Jackson ImmunoResearch Laboratories) were used for visualization. Stained samples were mounted in VECTASHIELD with DAPI mounting solution (Vector Laboratories).

### Cell-Cycle and Death Analyses

Cell viability was determined by the MTT assay as previously described (Twentyman and Luscombe, 1987). Apoptotic cell death

(E–G) Doxycycline-induced effects on the number (E), size (F), and percentage (G) of AP<sup>+</sup> colonies are abolished by the blockers specific for PI3K-AKT signaling. The colony-forming assays were performed by culturing dissociated H9 cells for 5 days in the presence of vehicle (DMSO, –) or doxycycline (1 μg/ml) with or without the PI3K-AKT blockers LY29002 (20 μM) or wortmannin (100 μM) or the RAF-ERK blocker PD98059 (10 μM). Significantly different from the control cultures treated with vehicle at \**p* < 0.001 and the cultures treated with doxycycline alone at #*p* < 0.05, *n* = 3 experiments per group, one-way ANOVA with Tukey post hoc analysis.

(H and I) PI3K enzyme activity enhanced by doxycycline. (H) Conventional autoradiograph-based PI3K activity assay (duplicated experiments). As a positive control for the assay, PI3K activities were determined, in parallel, in a control cell line (human breast cancer cells MDA-MB-231) with or without a reported PI3K inhibitor (eckol) (Hyun et al., 2011) (left panel). (I) The effect of doxycycline on PI3K activity was further assessed using a PI3 kinase activity/inhibitor assay kit. The assays were also carried out in the presence of other antibiotics or known PI3K inhibitors (controls). All error bars represent SEM of at least five independent experiments. Doxycycline (Dox); tetracycline (Tet); minocycline (Mino); kanamycin (Kan); chloramphenicol (Chlor); blasticidine (Blas). The graph depicts calculated PI3K enzyme activities after subtracting the activity without the inhibitor/activator. Inset, doxycycline dose-dependent PI3K activity curve. See also Figure S4.



was assessed with the percentage of cells positive for the apoptotic cell-specific markers cleaved Caspase-3 and Annexin V/PI by direct counting on microscope. Annexin V/PI-stained cells were further counted by using FACS (CantoII, BD Biosciences). FACS analysis was also applied for determining cell populations accumulated on each cell-cycle phase using FlowJo xV.0.7 software (Tree Star). Cell senescence was determined by the Senescence  $\beta$ -Galactosidase Staining Kit (Cell Signaling Technology) according to the manufacturer's instructions.

### Alkaline Phosphatase Activity Analysis

Alkaline Phosphatase Substrate kit I (Vector) was used to analyze alkaline phosphatase activity within clusters according to the manufacturer's instructions.

### Evaluation of Pluripotency

In vitro differentiation of the hESCs into the three germ layers was induced by withdrawing bFGF from feeder-free cultures for 2 weeks, after which cells were immunostained for markers specific for endoderm (FOXA2,  $\alpha$ -Fetoprotein [AFP]), mesoderm (smooth muscle actin [SMA], BRACHYURY), and ectoderm (TUJ1, GFAP). In vivo teratoma formation was induced by injecting  $1 \times 10^6$  H9 hESCs (cultured for 56 days with eight passages in the presence of doxycycline) under the gonad of 6-week-old NOD/SCID mice. Eight weeks later, the resulting tumors were removed and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E).

### Karyotyping

Karyotyping was carried out on hESCs (H9) maintained in the presence of doxycycline (1  $\mu$ g/ml) by standard G banding at the NEO-DIN Medical Institute (Seoul, Korea).

### Quantitative PCR Analyses

Total RNA preparation and cDNA synthesis were performed as previously described (Brederlau et al., 2006). High-throughput gene expression profiling was done for human stem cell transcriptional factors (cat. 330231 PAHS-501ZA) and cell apoptosis-related proteins (cat. 330231 PAHS-012ZA) using RT<sup>2</sup> Profiler PCR Array (QIAGEN). The results were further confirmed using conventional real-time PCR analysis (Park et al., 2005).

### Determination of Intracellular Signal Activation

Intracellular signals activated by doxycycline treatment were screened using Human Phospho-Kinase Array (Proteome Profiler Array, R&D Systems) and CBA multiplexed bead-based immunoassays (560005, 560010, 560012, 560013, 560015, 560059, 560065, 560093, 560150, 560792, BD) according to the manufacturers' protocols. Western blot analyses were performed as previously described (Chang et al., 2007) to confirm the results from the screens or to determine the levels of proteins, such as E-cadherin and pMLC, associated with actomyosin hyperactivation.

### PI3 Kinase Activity Assay

A conventional autoradiograph-based PI3K activity assay was carried out as previously described (Hyun et al., 2011). Briefly,

PI3K was immunoprecipitated with an anti-PI3K antibody (anti-p85) from total cell extracts from H9 hESCs that had been cultured in the presence or absence of doxycycline. Enzymatic reactions were initiated by adding [ $\gamma$ -<sup>32</sup>P]ATP and L- $\alpha$ -phosphatidylinositol (substrate) to the precipitates, and the radiolabeled product from the reaction was detected using autoradiography. The effects of doxycycline on PI3K activity were further determined using a commercially available PI3 kinase activity/inhibitor assay kit (Millipore), in which recombinant class I PI3K enzyme proteins react the substrate PIP2 in the absence of cell extracts. PI3K activator/inhibitor activities were calculated according to the manufacturer's instructions with some modifications.

### Cell Counting and Statistical Analysis

Immunoreactive cells (colonies) were counted in a whole area (96-well plate) or in uniform randomly chosen areas using an eyepiece grid at 200 $\times$  (24-well plate) or culture dishes with grid (6 cm) at 40 $\times$  or 100 $\times$ . Statistical comparisons between two groups were made by Student's two-tailed t test or two-tailed paired t test. One-way ANOVA with Tukey post hoc analysis (SPSS) was applied when more than two groups were compared. Data are expressed as mean  $\pm$  SEM.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2014.06.013>.

### AUTHOR CONTRIBUTIONS

M.-Y.C. and Y.-H.R. designed and performed experiments, analyzed data, and helped write the manuscript. S.-H.Y. carried out FACS and Phospho-Kinase Array analyses. S.-J.L. and R.-K.K. performed the PI3 kinase activity assays. H.K. performed the experiments to test doxycycline effects in mouse ESCs and teratoma formation assay. C.-H.P. gave technical support and conceptual advice. S.-H.L. funded the project, provided conceptual overview, designed and interpreted experimental results, and wrote the manuscript.

### ACKNOWLEDGMENTS

This work was supported by grants from the Bio & Medical Technology Development Program (2010-0020232), the Medical Research Center (2008-0062190), and the basic science research program (2012R1A1-A3010261), funded by the National Research Foundation of Korea (NRF) of the Ministry of Education, Science and Technology (MEST), Republic of Korea and the research fund of Hanyang university (HY-2014). We appreciate Dr. Suresh Ramakrishna and Myungjae Song for their technical supports.

Received: October 23, 2013

Revised: June 24, 2014

Accepted: June 25, 2014

Published: July 24, 2014



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