Stem Cell Reports
Article

ISSCR

OPEN ACCESS

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

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$^+$ 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 μg/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.

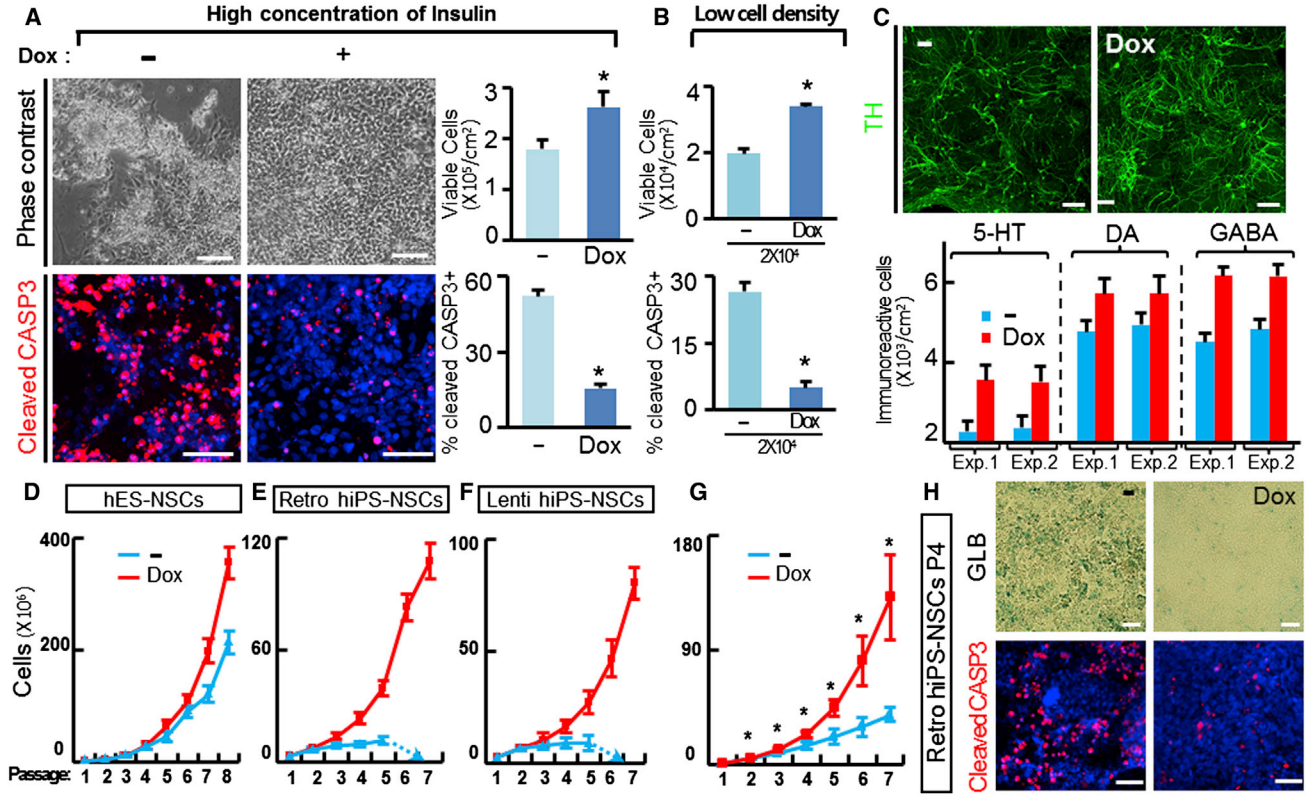

Figure 1. Effects of Doxycycline Supplementation on Cultures of hNSCs and Neurons Derived from hESC/iPSCs

(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 × 10^5 /cm^2 (A) or 2 × 10^4 /cm^2 (B) were cultured with or without doxycycline supplementation (1 μg/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 uM) are shown in the left panels. Scale bars, 50 μm.

(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 μm.

(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 β-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 μm. All data shown in this study are expressed as mean ± SEM.

See also Figure S1.

Doxycycline supplementation (1 μg/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