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Repair of Ischemic Injury by Pluripotent Stem Cell Based Cell Therapy without Teratoma through Selective Photosensitivity

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SUMMARY

Stem-toxic small molecules have been developed to induce selective cell death of pluripotent stem cells (PSCs) to lower the risk of teratoma formation. However, despite their high efficacies, chemical-based approaches may carry unexpected toxicities on specific differentiated cell types. Herein, we took advantage of KillerRed (KR) as a suicide gene, to selectively induce phototoxicity using visible light via the production of reactive oxygen species. PSCs in an undifferentiated state that exclusively expressed KR (KR-PSCs) were eliminated by a single exposure to visible light. This highly selective cell death in KR-PSCs was exploited to successfully inhibit teratoma formation. In particular, endothelial cells from KR-mPSCs remained fully functional in vitro and sufficient to repair ischemic injury in vivo regardless of light exposure, suggesting that a genetic approach in which KR is expressed in a tightly controlled manner would be a viable strategy to inhibit teratoma formation for future safe PSC-based therapies.

INTRODUCTION

For more than a decade, multipotent stem cells have been extensively studied for various cardiovascular diseases such as heart failure and ischemic cardiomyopathy, which was defined as the "first-generation" cell therapies (Behfar et al., 2014). The "next-generation" cell therapies include pluripotent stem cells (PSCs) such as human embryonic stem cells (hESCs) and induced PSCs (iPSCs) in an effort to maximize therapeutic value of stem cells for cardiovascular diseases (Ban et al., 2014).

However, PSCs have major safety concerns, such as teratoma formation due to possible contamination of the residual undifferentiated PSCs (Lee et al., 2013a). Teratoma is a unique characteristic of PSCs owing to their pluripotency and ability to undergo unlimited proliferation (Gruen and Grabel, 2006). These properties (e.g., pluripotency and limitless proliferation), which theoretically allow the sufficient supply of cardiomyocytes, cardiac progenitors, and endothelial cells, make PSCs the most promising cell source for regenerative medicine (Robinton and Daley, 2012). The unlimited proliferation capacity of PSCs is similar to the proliferation of cancer cells, which results from high telomerase activity (Thomson et al., 1998) and constant inhibition of retinoblastoma protein (Burdon et al., 2002). Therefore, the presence of a few undifferentiated PSCs or incompletely differentiated cells that escape the strict sorting process would be a high risk factor for tumor development in safe stem cell therapies (Masuda et al., 2014).

However, teratoma or tumor formation following xenotransplantation of human (h)PSCs into animal models (mostly rodents) has been barely reported (Kriks et al., 2011; Laflamme et al., 2007), with a few exceptions (Brederlau et al., 2006; Seminatore et al., 2010). A recent study by Doi et al. (2012) demonstrated that extended differentiation of hPSCs reduces the risk of teratoma formation in a primate model. Therefore, stringent selection of differentiated cells by flow cytometry for selective sorting (Cho et al., 2007) or prolonged differentiation (Brederlau et al., 2006; Zhang et al., 2001) would be sufficient to avoid tumorigenicity. Nevertheless, considering the "host-dependent tumorigenicity" of ESCs (e.g., xenotransplantation of hPSCs into a rodent model) (Erdö et al., 2003), the risk of teratoma formation by hPSCs in humans remains to be resolved, especially given that several clinical trials of hPSC-based therapies are ongoing (Cyranoski, 2013). Not only mouse embryonic bodies (mEBs), but also differentiated cells from mouse embryonic stem cells (mESCs) can reportedly form teratomas when transplanted into a rodent model, even after stringent cell sorting (Arnhold et al., 2004; Fujikawa et al., 2005; Moon et al., 2013).

A number of approaches have been developed to reduce the risk of teratoma formation, including antibody-based selective elimination (Tang et al., 2011), small molecules (Ben-David et al., 2013; Lee et al., 2013b), and integration of a suicide gene (Li and Xiang, 2013). Among them, the use of various chemicals to achieve selective cell death of PSCs (referred as "stemotoxic" in a review, see Knoepfler,

2009) may have unexpected caveats, despite being highly efficient (Knoepfler, 2009). Additionally, there is no evidence indicating that such chemical treatment ensures functionality of differentiated cells in vivo (Masuda et al., 2014). Therefore, we attempted to eliminate undifferentiated PSCs without using small molecules and reveal that the differentiated cells after selective ablation of undifferentiated PSCs remain fully functional in vivo. To this end, KillerRed (KR), an artificial photosensitizer protein derived from a hydrozoan chromoprotein (Bulina et al., 2006) which produces reactive oxygen species (ROS) when exposed to visible light of 540–580 nm (Wang et al., 2012), was introduced into PSCs with a pluripotent-specific promoter such that it was only expressed in undifferentiated PSCs. A single treatment with visible light successfully eliminated KR-expressing mouse PSCs (KR-mPSCs) in an ROS-dependent manner, while endothelial cells (ECs) differentiated from KR-mPSCs survived and remained functional in vitro and in vivo. The similar results were repeated in a human ESC (hESC) model. Importantly, KR-mPSCs did not form teratomas after being exposed to the light, whereas ECs differentiated from KR-mPSCs effectively regenerated blood vessels under ischemic conditions in a rodent model. Our results strongly support and provide the "proof of concept" that a KR-based suicide gene approach in which visible light selectively induces phototoxicity in PSCs would be an efficient strategy to reduce the risk of tumorigenicity in PSC-based cardiovascular repair.

RESULTS

Establishment of KR-mPSCs

We aimed to induce selective cell death of undifferentiated PSCs using visible light, which is supposedly harmless to normal cells. We sought to specifically express KR, a photosensitizer protein, in a pluripotency-dependent manner. To this end, we took advantage of previously developed pluripotency-specific EOS (early transposon promoter and *OCT-4* and *SOX2* enhancers) vector systems such as EOS-C(3+) (Hotta et al., 2009), which contains multimerized *OCT-4* core enhancer element conserved region 4 (CR4) (Okumura-Nakanishi et al., 2005). KR expression was designed to localize to mitochondria by adding a mitochondrial-targeting sequence (MTS) (Figures 1A and 1B) to maximize the induction of cell death via the production of ROS (Rizzuto et al., 1995).

The constructed plasmid (EOS-C(3+)-KR) was delivered to J1 mESC line (J1), and KR expressing mESCs were sorted based on their red fluorescence (Figure 1C). KR-mESCs formed teratomas consisting of ectodermal, mesodermal, and endodermal tissues, suggesting that the introduction of KR did not interfere with the pluripotency of mESCs (Figure S1A). Similarly, EOS-C(3+)-KR was introduced into reprogrammable mouse embryonic fibroblasts (MEFs) (Carey et al., 2010) and the cells were subsequently reprogrammed by doxycycline treatment (Figure 1D). Consistent to previous reports (Hotta et al., 2009), the fully reprogrammed cells were readily distinguished by their red fluorescence, owing to activation of the EOS promoter as pluripotency was acquired, in comparison with non-fluorescent control mouse iPSCs (miPSCs) (Figures 1D, 1E, and S1B). The established miPSCs expressing EOS-C(3+)-KR (KR-miPSCs) formed teratomas, similar to KR-mESCs (Figure S1C). While several typical pluripotency markers were equivalently expressed in KR-mESCs and KR-miPSCs compared with their parental cells, KR was exclusively expressed in KR-mPSCs (Figures 1F and 1G). These results indicate that the introduction of KR did not impair the pluripotency properties of PSCs.

Specific Expression of KR in a Pluripotent-Dependent Manner

Red fluorescence from KR expression driven by activation of the EOS-C(3+) promoter was distinct to KR-mPSCs (Figures 1C–1E); therefore, we next investigated whether expression of KR was suppressed as KR-mESCs underwent differentiation. Spontaneous differentiation of KR-mESCs was induced through embryonic body (EB) formation followed by monolayer culture (Figure 2A, inserted). Red fluorescence from KR was observed in the EB and gradually diminished over time (Figure 2A). Consistently, *KR* expression was suppressed concurrent with *Nanog* and *Oct-4* suppression during spontaneous differentiation (Figure 2B). KR protein level was also markedly suppressed during differentiation (Figure S2A). Expression of marker genes of all three germ layers (*Fgf5*: ectoderm, *Brachyury*: mesoderm, and *Sox17*: endoderm) clearly increased in differentiated cells (after 6 days of differentiation) (Figure 2B). Similarly, red fluorescence from KR expression was only observed in OCT-4-positive, not in OCT-4-negative populations (cultured in a monolayer for 4 days after EB formation), indicating that KR was specifically expressed in undifferentiated PSCs (Figure 2C, white dotted line).

Induction of PSC-Specific Cell Death by Visible Light

KR expression was tightly controlled in a pluripotency-specific manner (Figure 2); therefore, irradiation with visible light of 540–580 nm was expected to induce cell death specifically in KR-mPSCs, not in differentiated cells. KR-mESCs displaying red fluorescence were completely eliminated within 6 hr after a single exposure to green light (1.3 W/cm^2, 540 nm), whereas control mESCs that did not express KR had no apparent alteration (Figure 3A). The phototoxic effects in KR-mESCs were quantified by