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Figure Legends

Figure 1. *In vitro* imaging and differentiation of NS/PCs

(A) The *ff*Luc-cp156 reporter transgene. (B) Phase contrast and fluorescence images of the Venus-positive neurospheres derived from fetal ganglionic eminence at embryonic day 14.5. (C) There is a positive linear relationship between cell number and photon count in bioluminescence imaging (BLI). After passage 3, neurospheres had differentiated into β-III tubulin (Tuj-1)-positive neurons (E), glial fibrillary acid protein (GFAP)-positive astrocytes (F) and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase)-positive oligodendrocytes (G). (H) Quantitative analysis of the differentiated neural stem/progenitor cells (NS/PC) phenotypes. n=3. Scale bars; 100 μm in (B and C), 50 μm in (D–F).

Figure 2. BLI analysis

NS/PCs were transplanted into the lesion epicenter (E group) or regions 1 mm rostral and caudal of the rim of the lesion (RC group) at 9 days post-injury (dpi). Representative bioluminescence images of mice in the E group (A) and the RC group (B) at 7 and 35 days post-transplantation (dpt). (C) Quantitative analysis of the bioluminescence images revealed that there was no significant difference in the photon counts between the E and RC groups. The E and RC groups were subdivided into 'High' and 'Low' groups according to the number of NS/PCs transplanted. The mean photon counts of the E-High and E-Low groups (D), and the RC-High and RC-Low groups (E) were similar at 35 dpt.

Figure 3. Distribution and differentiation of grafted NS/PCs

(A) Anti-green fluorescent protein (GFP) DAB staining of sagittal sections of the spinal cord at 42 dpt. Grafted NS/PCs were outside the lesion epicenter in both the E and RC groups. (B) Immunohistochemical analysis showed the grafted NS/PCs had differentiated into Hu-positive

neurons, GFAP-positive astrocytes and adenomatous polyposis coli antigen (APC)-positive oligodendrocytes in both the E and RC groups. (C) Quantitative analysis revealed that there were significant differences (*$p < 0.05$) in the percentage of neurons and astrocytes between the E and RC groups. n=3. Scale bars; 1000 μm in (A), 10 μm in (B).

Figure 4. Motor functional recovery

The locomotor function of the hind limbs was assessed by the Basso mouse scale (BMS). (A) Motor functional recovery was significantly better (*$p < 0.05$, **$p < 0.01$, RC versus PBS; †$p < 0.05$, ††$p < 0.01$, E versus PBS) in the E and RC groups than in the phosphate buffered saline (PBS) group. There was no significant difference between the E and RC groups. (B) The E and RC groups were subdivided into 'High' and 'Low' groups according to the number of NS/PCs transplanted. There was no significant difference in BMS scores among these four groups. BMS scores were significant higher in each of these four groups than in the PBS group at 49 dpi.

Figure 5. Evaluation of NF-H staining

(A–C) Anti-neurofilament 200 kDa (NF-H) DAB staining of sagittal sections of the spinal cord on 42 dpt. NF-H-positive areas were quantified in the rostral, epicenter and caudal regions. (D) NF-H-positive areas in each of these regions were significantly larger in the E and RC groups than in the PBS group, whereas there was no significant difference (*$p < 0.05$) between the E and RC groups. Scale bars; 1000 μm.

Figure 6. Evaluation of PECAM-1 staining

(A–C) Anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) DAB staining of sagittal sections of spinal cord on the 42 dpt. PECAM-1-positive areas were quantified in the rostral, epicenter

and caudal regions. (D) The PECAM-1-positive areas in each of these regions were significantly larger (*$p < 0.05$) in the E and RC groups than in the PBS group, whereas there was no significant difference between the E and RC groups. Scale bars; 1000 μm.

Figure 7. qRT-PCR analysis of growth factors, inflammatory cytokines, markers of inflammatory cells and BMPs

The E and RC segments of mice with contusive spinal cord injury were sampled at 9 dpi. mRNA expression was assessed by qRT-PCR. Brain-derived neurotrophic factor (Bdnf) expression was significantly higher (*$p < 0.05$) in the RC segment than in the E segment. The expression levels of the other growth factors, inflammatory cytokines, markers of inflammatory cells and bone morphogenetic proteins (BMPs) did not significantly differ between the E and RC segments.

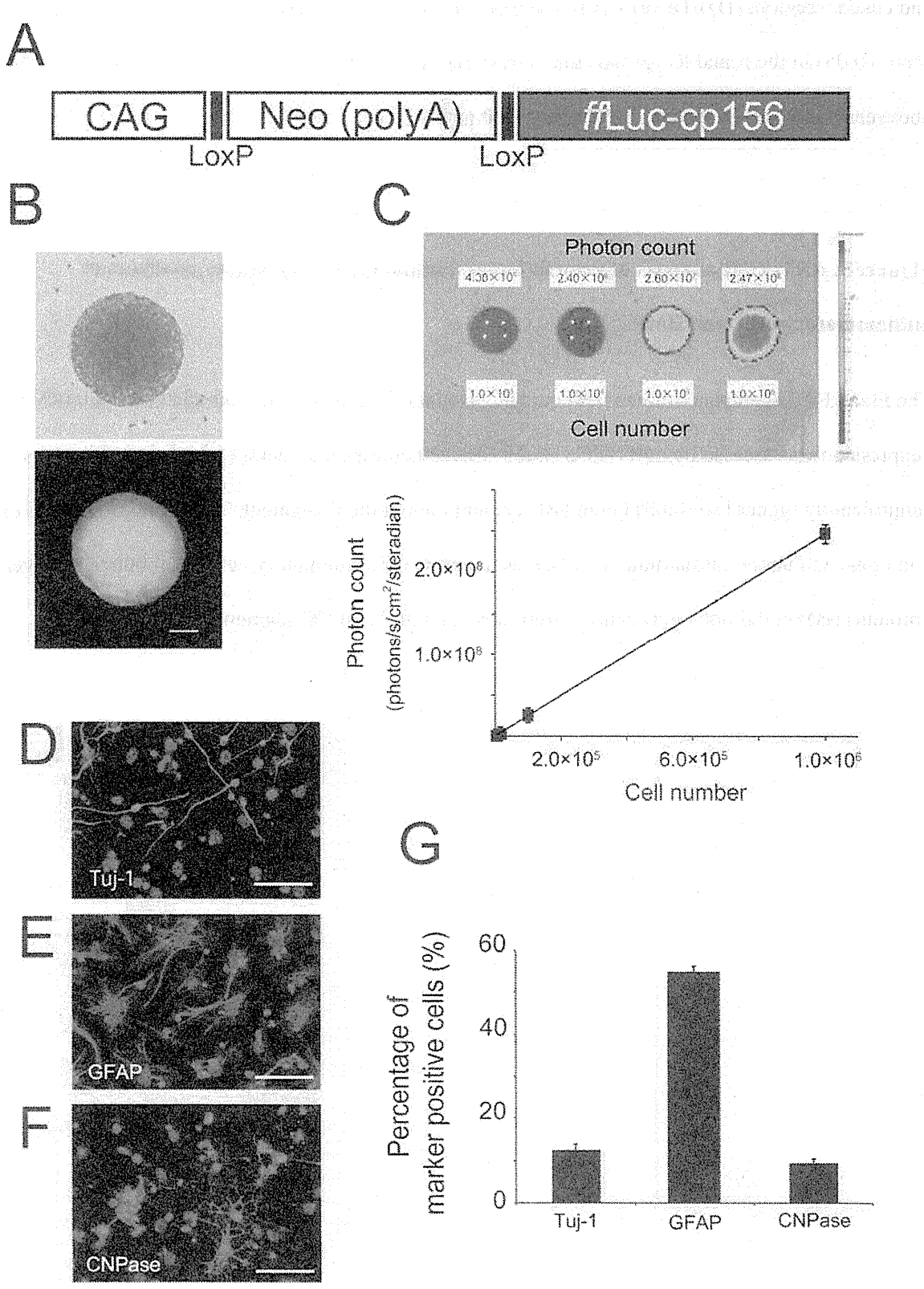
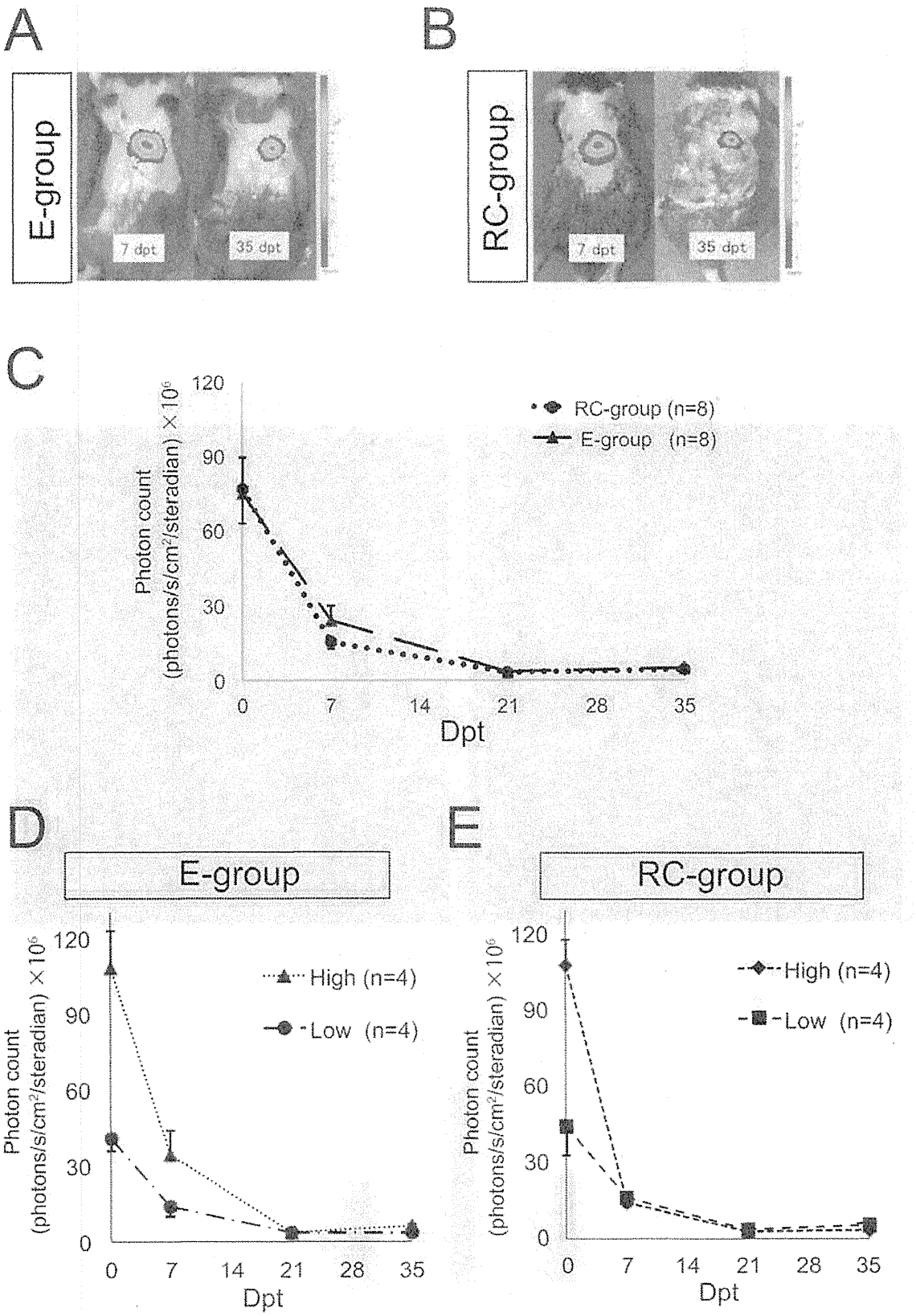


FIGURE 1



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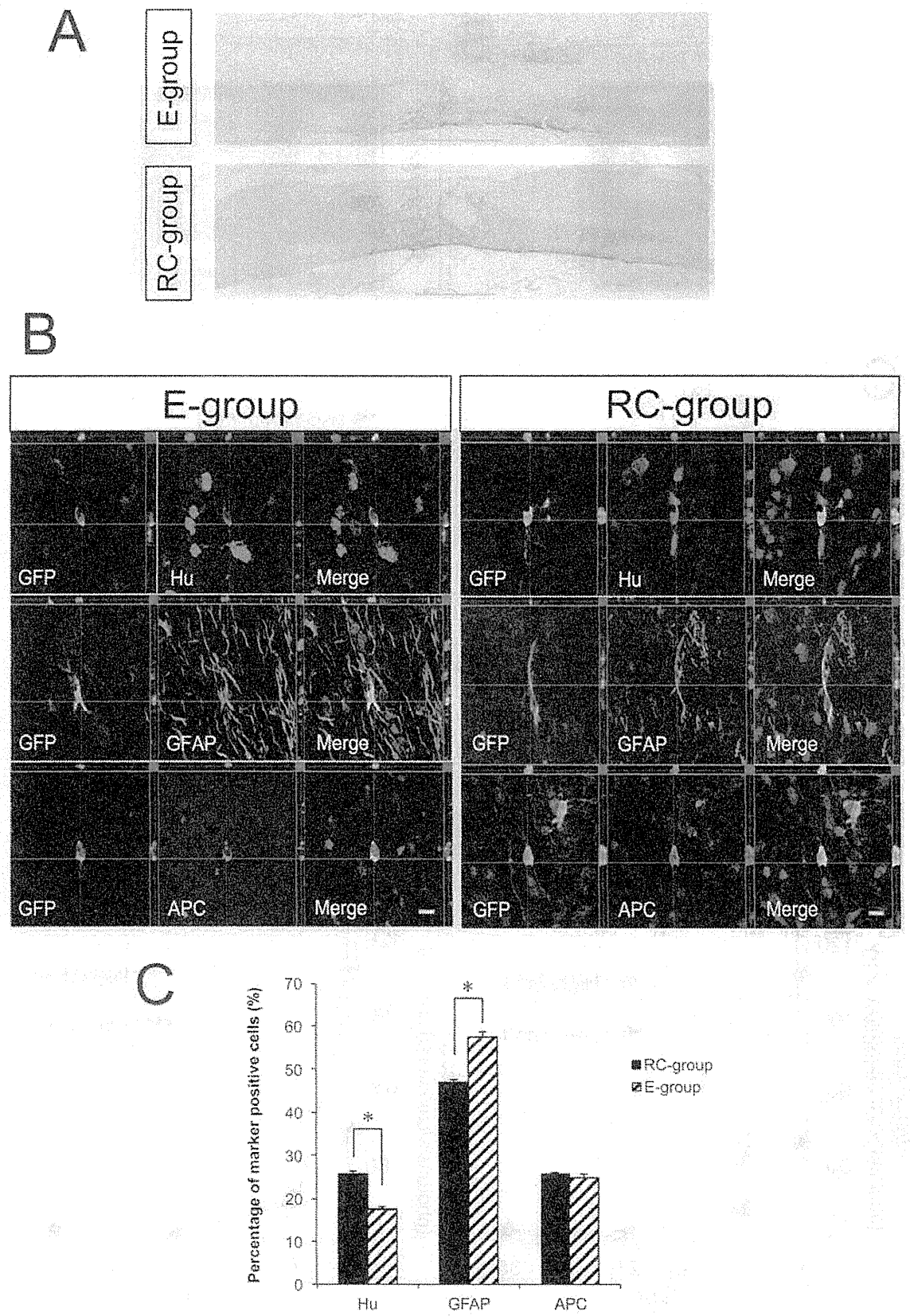

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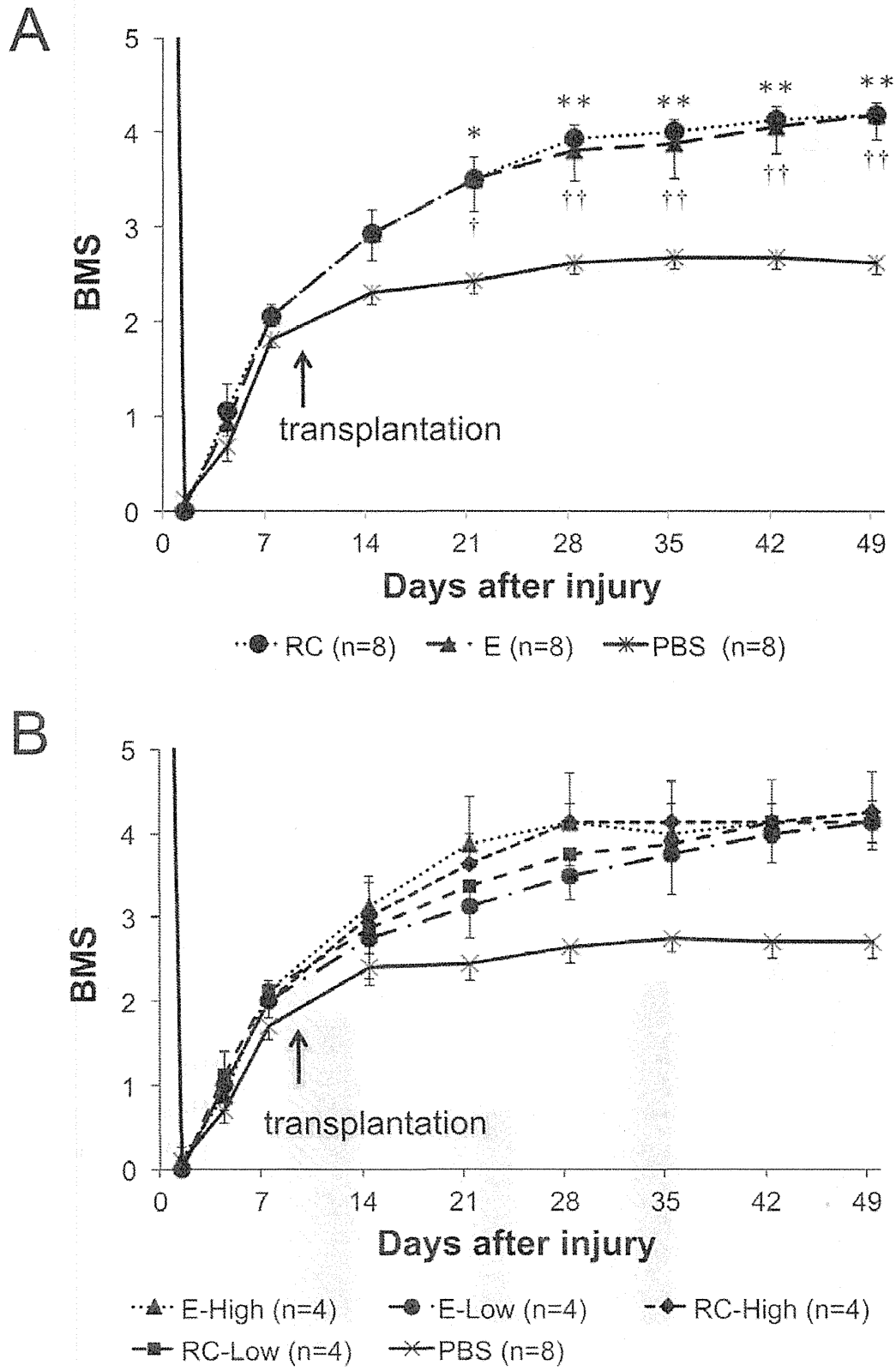

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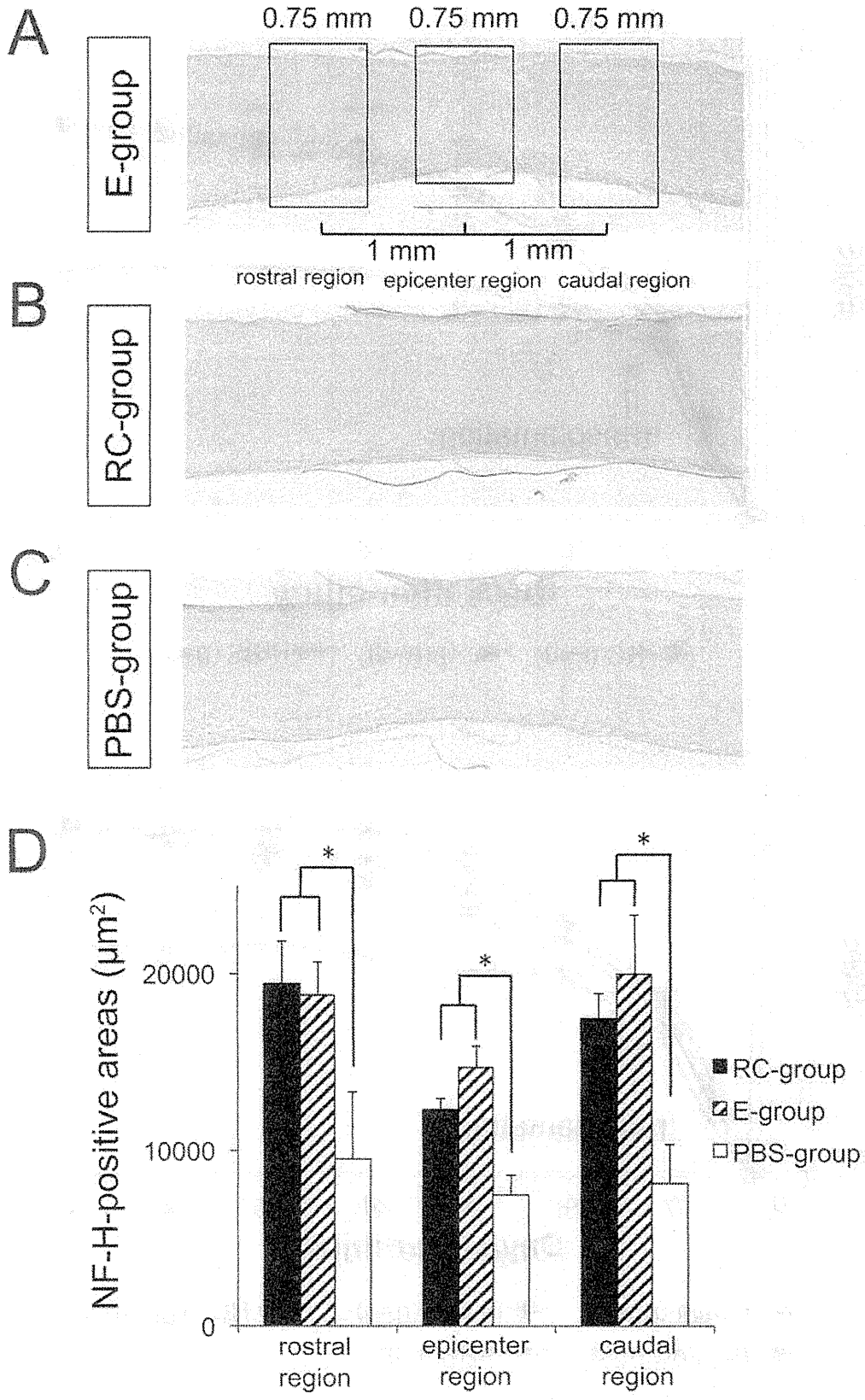

FIGURE 5

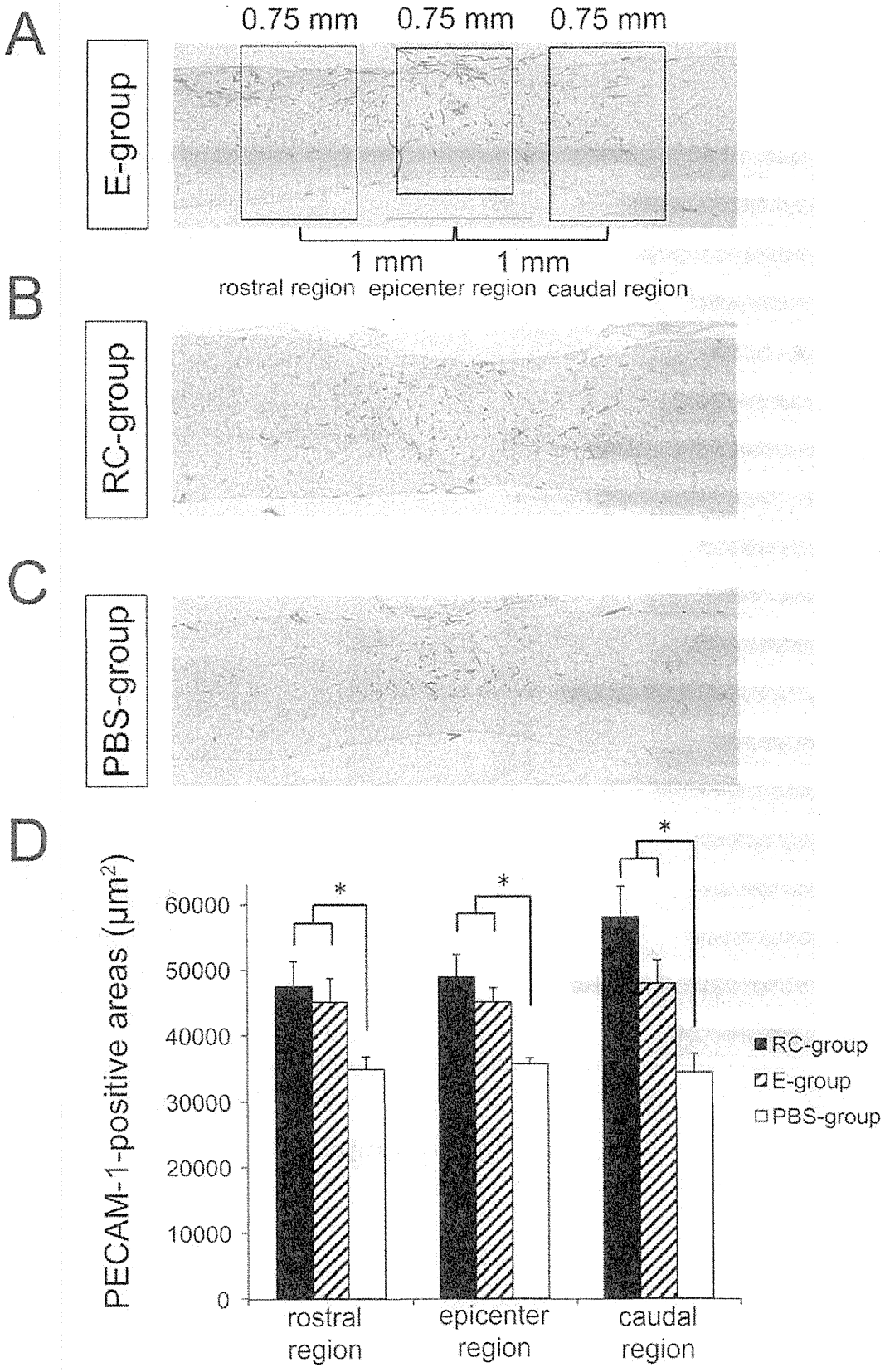

FIGURE 6

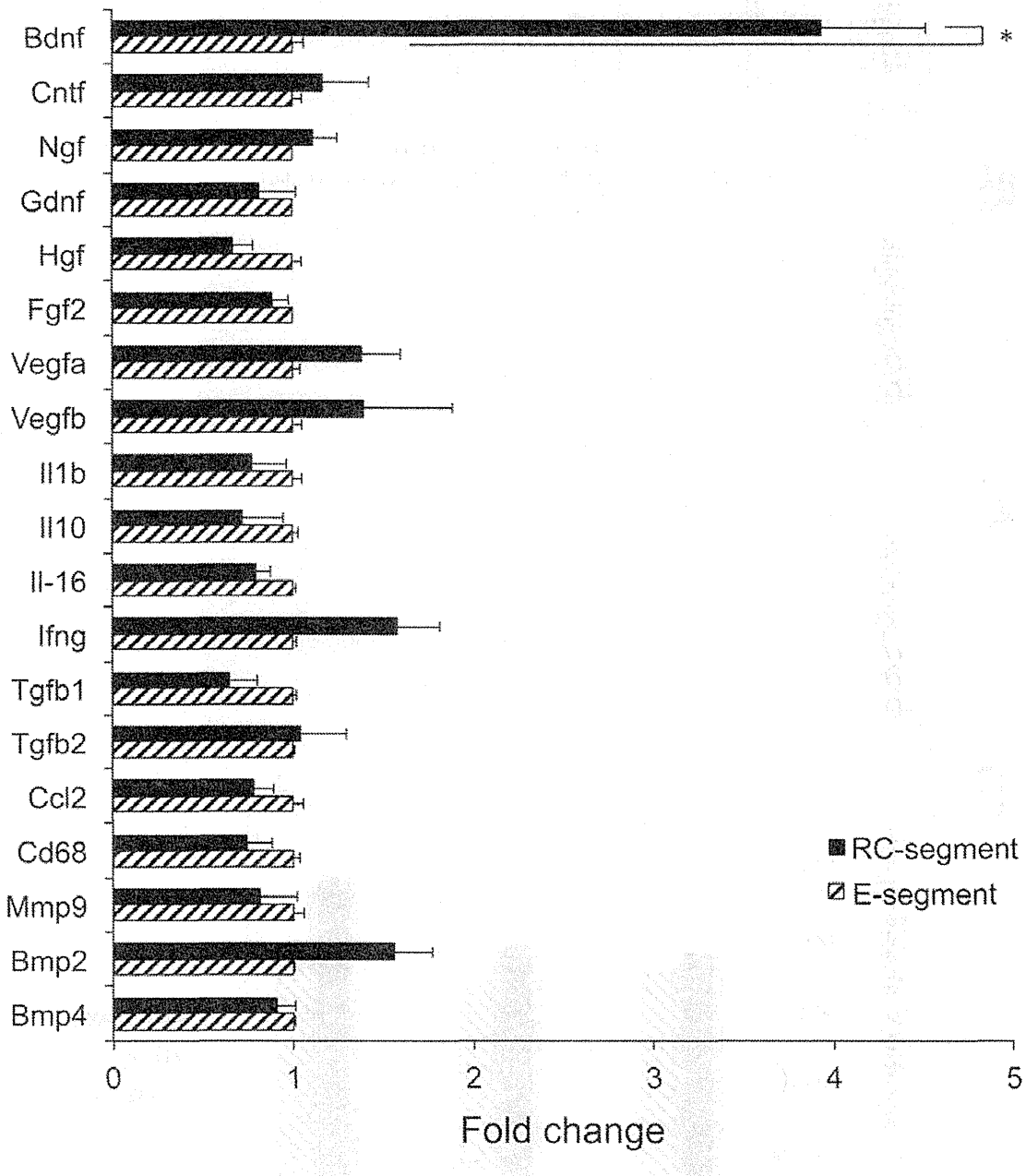

FIGURE 7

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American Heart Association.

Steps Toward Safe Cell Therapy Using Induced Pluripotent Stem Cells

Hideyuki Okano, Masaya Nakamura, Kenji Yoshida, Yohei Okada, Osahiko Tsuji, Satoshi Nori, Eiji Ikeda, Shinya Yamanaka and Kyoko Miura

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This article is the first in a new thematic series on **Recent Advances in iPS Cell Research**, which includes the following articles:

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Differentiation of iPSCs Into Mature Myocytes

Epigenetic Reprogramming for Cardiovascular Regeneration

Shinya Yamanaka, Guest Editor

Steps Toward Safe Cell Therapy Using Induced Pluripotent Stem Cells

Hideyuki Okano, Masaya Nakamura, Kenji Yoshida, Yohei Okada, Osahiko Tsuji, Satoshi Nori, Eiji Ikeda, Shinya Yamanaka, Kyoko Miura

Abstract: **The enthusiasm for producing patient-specific human embryonic stem cells using somatic nuclear transfer has somewhat abated in recent years because of ethical, technical, and political concerns. However, the interest in generating induced pluripotent stem cells (iPSCs), in which pluripotency can be obtained by transcription factor transduction of various somatic cells, has rapidly increased. Human iPSCs are anticipated to open enormous opportunities in the biomedical sciences in terms of cell therapies for regenerative medicine and stem cell modeling of human disease. On the other hand, recent reports have emphasized the pitfalls of iPSC technology, including the potential for genetic and epigenetic abnormalities, tumorigenicity, and immunogenicity of transplanted cells. These constitute serious safety-related concerns for iPSC-based cell therapy. However, preclinical data supporting the safety and efficacy of iPSCs are also accumulating. In this Review, recent achievements and future tasks for safe iPSC-based cell therapy are summarized, using regenerative medicine for repair strategies in the damaged central nervous system (CNS) as a model. Insights on safety and preclinical use of iPSCs in cardiovascular repair model are also discussed.** (*Circ Res.* **2013;112:523-533.)**

Key Words: induced pluripotent stem cell ■ neural stem/progenitor cell ■ transplantation ■ spinal cord injury

Applications for the Use of Induced Pluripotent Stem Cells and Related Concerns: An Overview

Ever since pioneering reports introduced mouse[1] and human-induced[2–4] pluripotent stem cells (iPSCs) to the scientific community and the populace at large, there has been an increasing interest in applications for their use in the fields of biomedical research. These include cell therapy in regenerative medicine and modeling of human disease. By characterizing

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Nonstandard Abbreviations and Acronyms	
CNPase	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS	central nervous system
EB	embryoid body
ESC	embryonic stem cell
GFAP	glial fibrillary acidic protein
hiPSC	human iPSC
iPSC	induced pluripotent stem cell
PNS	primary neurosphere
NS/PC	neural stem/progenitor cell
SCI	spinal cord injury
SNS	secondary neurosphere

the in vitro phenotype of disease-specific iPSC-derived cells, researchers have gained new insights, not only into the pathophysiology of the particular disorder, but also into strategies for drug screening and the development of novel therapeutic agents.[5] Although disease modeling is emerging as an extremely exciting research field, this issue will not be discussed here because of space limitations. In regard to the application of iPSCs for regenerative medicine, increasing experimental evidence supports their therapeutic benefits.[6] However, some recent reports also indicate risk factors for the use of iPSCs, such as genetic and epigenetic abnormalities that could take place during reprogramming or maintenance in subsequent cell culture.[7–11] Of particular relevance is the potential tumorigenicity[6,12–14] and immunogenicity[15] associated with iPSC-based cell therapy. The purpose of this review article is to summarize previous efforts in the field, as well as the current status of iPSC-based cell therapy. Work from this group that uses iPSC-derived neural stem/progenitor cells (NS/PCs) in preclinical studies for repair of the damaged central nervous system (CNS) is discussed, with a special emphasis on spinal cord injury (SCI). These findings and those from other groups are presented in light of an important task for the future: how should researchers best address the implicit pitfalls of iPSC-based cell therapy?

Early Development of iPSC-Based Technologies

Mouse iPSCs were first established by Takahashi and Yamanaka in 2006,[1] and continuous progress has been made in the methods for their production during recent years. This article does not attempt to fully cover the methodological details of iPSC production; however, the key findings that are related to safety issues of iPSC-based cell therapy are summarized herein.

Initially, iPSCs were generated from mouse fibroblasts by retroviral introduction of the transcription factors *Oct4*, *Sox2*, *c-Myc*, and *Klf4*.[1] The actions of these 4 transcription factors are thought to reprogram somatic cells, including fibroblasts, into embryonic stem cell (ESC)-like pluripotent cells through multiple stochastic epigenetic events (eg, silencing of somatic genes and retroviral genes that putatively activate Dnmt3a and 3b) and activation of various pluripotent genes.[16] The original mouse iPSCs were selected for the expression of *Fbxo15*, a marker of undifferentiated ES cells, and thus are called *Fbxo15*-iPSCs. These cells demonstrated in vitro and in vivo differentiation into various types of cells from all 3 germ layers, but their epigenetic and biological properties differed from ESCs.[1,16] *Fbxo15*-iPSCs are currently understood to be partially reprogrammed iPSCs in which the retroviral transgenes are still expressed and the autoregulatory loops of endogenous *Oct4/Sox2/Nanog* genes are not completely established.[16] In fact, our group could not obtain pups from *Fbxo15*-iPSC-derived chimeric mice, although these cells contributed to embryonic development.[1]

In July 2007, 2 groups independently reported that selection for *Nanog* or *Oct4* expression resulted in germline-competent mouse iPSCs with increased mouse ESC-like gene expression and DNA methylation patterns compared with *Fbxo15*-iPS cells.[17,18] *Nanog* and *Oct4* are known to be crucial for the maintenance of undifferentiated ESCs by forming autoregulatory loops of endogenous *Oct4/Sox2/Nanog* genes.[16] *Nanog*-iPSCs were generated by Okita et al[17] from fibroblasts of transgenic mice containing the *Nanog*-GFP-IRES-Puro^r reporter construct. Fibroblasts from this transgenic mouse do not express the endogenous *Nanog* gene; hence, they are puromycin sensitive. However, by the retroviral transduction of *Oct4*, *Sox2*, *c-Myc*, and *Klf4*, these fibroblasts are reprogrammed into ESC-like cells and start to express the protein products of the endogenous *Nanog* gene, as well the as the *Nanog*-GFP-IRES-Puro^r reporter gene. As such, the cells acquire puromycin resistance and green fluorescent protein expression after transduction. Thus, through selection of puromycin-resistant and green fluorescent protein-positive colonies, iPSC clones with high endogenous *Nanog* expression can be obtained. These reprogrammed *Nanog*-iPSCs, as well as *Oct4*-iPSCs, are likely to be at a more advanced state of development than *Fbxo15*-iPSCs, with a phenotype characterized by activated Dnmt3a and 3b, silenced retroviral transgenes, and established autoregulatory loops of the *Oct4/Sox2/Nanog* genes. However, there is no guarantee that *Nanog*-selected iPSCs or *Oct4*-selected iPSCs are completely equivalent to mouse ESCs.[16] In fact, reactivation of the *c-Myc* retroviral transgene increased tumorigenicity in chimeric mice obtained via blastocyst injection of *Nanog*-selected iPSCs.

In January 2008, Nakagawa et al[19] reported a modified protocol for the generation of mouse iPSCs that did not require the *c-Myc* transgene. Importantly, chimeric mice derived from the *c-Myc*-minus iPSCs did not develop tumors during the study period. Furthermore, the omission of the *c-Myc* transgene resulted in the efficient isolation of iPSCs without drug selection (eg, *Nanog*-puromycin selection). This finding is advantageous for the generation of human iPSCs (hiPSCs), because the human genome does not accommodate the *Nanog*-green fluorescent protein-IRES-Puro^r reporter gene (Figure 1).

From April 2008 to February 2009, 3 articles were published reporting that mouse iPSCs could be generated from cells of various adult somatic origins, including terminally differentiated B lymphocytes,[20] liver and stomach cells,[21] and neural stem cells,[22] although the required combinations of transgenes were different depending on the somatic origin. Thus, the epigenetic state of the somatic cells affected the efficacy of their reprogramming into iPSCs.