

# Wnt/ $\beta$ -Catenin Signaling Triggers Neuron Reprogramming and Regeneration in the Mouse Retina

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## SUMMARY

Cell-fusion-mediated somatic-cell reprogramming can be induced in culture; however, whether this process occurs in mammalian tissues remains enigmatic. Here, we show that upon activation of Wnt/ $\beta$ -catenin signaling, mouse retinal neurons can be transiently reprogrammed in vivo back to a precursor stage. This occurs after their spontaneous fusion with transplanted hematopoietic stem and progenitor cells (HSPCs). Moreover, we demonstrate that retinal damage is essential for cell-hybrid formation in vivo. Newly formed hybrids can proliferate, commit to differentiation toward a neuroectodermal lineage, and finally develop into terminally differentiated neurons. This results in partial regeneration of the damaged retinal tissue, with functional rescue. Following retinal damage and induction of Wnt/ $\beta$ -catenin signaling, cell-fusion-mediated reprogramming also occurs after endogenous recruitment of bone-marrow-derived cells in the eyes. Our data demonstrate that in vivo reprogramming of terminally differentiated retinal neurons after their fusion with HSPCs is a potential mechanism for tissue regeneration.

## INTRODUCTION

The nucleus of a differentiated cell can be reprogrammed in vitro back to a pluripotent phenotype by ectopic overexpression of some defined stem cell factors, by nuclear transfer, or by cell fusion with embryonic stem cells (ESCs). Modulation of signaling pathways such as those of Wnt/ $\beta$ -catenin, MAPK/ERK, and PI3K/Akt can strikingly enhance reprogramming activity (Lluis and Cosma, 2009, 2010). Cell fusion is a well-known mechanism to change the potency of differentiated cells in vitro (Blau et al., 1983, 1985). Polyethylene glycol (PEG)-mediated embryonic-

germ/thymocyte or ESC/thymocyte hybrids show reactivation of the Oct4-GFP transgene as well as erasure of DNA methylation associated with imprinted genes and reactivation of the inactive X chromosome. Notably, hybrid cells can differentiate toward the three germ layers (Tada et al., 1997; Tada et al., 2001). Similarly, human fibroblasts can be reprogrammed to pluripotency in hybrids formed with human ESCs (Cowan et al., 2005).

Remarkably, coculture of ESCs with neural stem cells or with bone marrow cells (BMCs) leads to spontaneous formation of tetraploid hybrids with a pluripotent phenotype (Terada et al., 2002; Ying et al., 2002). We reported that the periodic accumulation of  $\beta$ -catenin in the ESC nucleus up to a specific threshold enhances the efficiency of ESCs to reprogram somatic cells after fusion (Lluis et al., 2008). Of note, Wnt-dependent reprogramming occurs efficiently also after spontaneous cell-cell fusion (Lluis et al., 2010).

Cell-cell fusion is a physiological mechanism that controls developmental processes, such as fertilization and muscle and bone development (Sullivan and Eggan, 2006); moreover, it can also occur in vivo after bone marrow (BM) transplantation, both in mice and in humans. Indeed, transplanted hematopoietic cells can fuse to hepatocytes, cardiomyocytes, and Purkinje cells, which leads to the formation of stable heterokaryons and synkaryons (i.e., hybrids with one or two nuclei), in different organs (Ogle et al., 2005). Hematopoietic cells can naturally fuse with human Purkinje cells in vivo (Weimann et al., 2003). These fusion events have been shown to increase after injury and inflammation, such as in the case of fusion between BM-derived cells and Purkinje neurons in the cerebellum of mice (Johansson et al., 2008) or in the case of fusion of lymphoid and myeloid blood cells with cells from different tissues (Nygren et al., 2008).

These findings prompted us to determine whether stem and progenitor cells (SPCs) can fuse with retinal neurons and Müller glia after their transplantation in damaged retinas and whether the in-vivo-formed hybrids can undergo reprogramming. Furthermore, our aim was to investigate whether activation of Wnt/ $\beta$ -catenin signaling pathway can trigger the reprogramming process in vivo. Finally, we wanted to investigate whether

cell-fusion-mediated reprogramming is a mechanism of nervous tissue regeneration.

Wnt-secreted glycoproteins are morphogens that control a variety of different developmental processes and cell homeostasis mechanisms, such as cell proliferation, cell polarity, stem cell self-renewal, and cell-fate determination, in embryonic life (Logan and Nusse, 2004). After binding of Wnt ligands with receptor, Frizzled (Fz), and coreceptor, lipoprotein-receptor-related proteins 6 and 5 (LRP6/5), the  $\beta$ -catenin destruction complex is inactivated. The destruction complex includes the tumor suppressor adenomatous polyposis coli (APC), the scaffold protein Axin2, casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). Its function is to phosphorylate  $\beta$ -catenin through CK1 and GSK3. After that, the phosphorylated  $\beta$ -catenin can be ubiquitinated and degraded via the activity of the proteasome (MacDonald et al., 2009). In a recent study, it was shown that Wnt signaling does not induce inactivation of the destruction complex but rather suppresses  $\beta$ -catenin ubiquitination (Li et al., 2012). In both models, after Wnt binding to its receptor,  $\beta$ -catenin is stabilized and can translocate into the nucleus, where it can activate target genes.

To test our hypothesis, we choose the eye as model system. Notably, the eye is an isolated organ that shows low immune responses to cells and viral vectors (Streilein et al., 2002); in addition, the confined space allows injection of a small number of cells. Moreover, the retina is the most accessible area of the CNS, and we wanted to determine whether neurons can undergo fusion with BM-derived cells and reprogramming.

Here, we show that upon retinal damage induced by injection of the glutamate receptor antagonist N-methyl-D-aspartate (NMDA) into the vitreous, transplanted SPCs, such as mouse hematopoietic stem and progenitor cells (mHSPCs), human (h) HSPCs, retinal (R)SPCs, and ESCs, can fuse with retinal neurons in vivo with high efficiency. Importantly, we show that the fate of these hybrids is to embark upon apoptosis unless Wnt/ $\beta$ -catenin signaling is activated in the transplanted cells. Indeed, the activation of the Wnt pathway induces reprogramming of retinal neurons back to precursor or embryonic stages after HSPC or ESC fusion, respectively. HSPC-derived reprogrammed hybrids can proliferate and in turn differentiate into ganglion and amacrine neurons, thereby contributing to retinal regeneration. Remarkably, multielectrode recordings of retinal explants showed functional rescue of ganglion neurons to light response in the regenerated retinas. Finally, we also show fusion and reprogramming of retinal neurons after endogenous recruitment of BMCs in damaged retinas.

In conclusion, we show here that upon NMDA-induced retinal damage, Wnt/ $\beta$ -catenin-mediated reprogramming of terminally differentiated cells, such as retinal neurons, can occur in vivo. This represents a way to promote cell and tissue regeneration in mammals.

## RESULTS

### NMDA-Induced Injury Triggers Fusion between Retinal Neurons and SPCs

Following BM transplantation, hematopoietic cells can fuse to different somatic cells in vivo, which can contribute to the repair

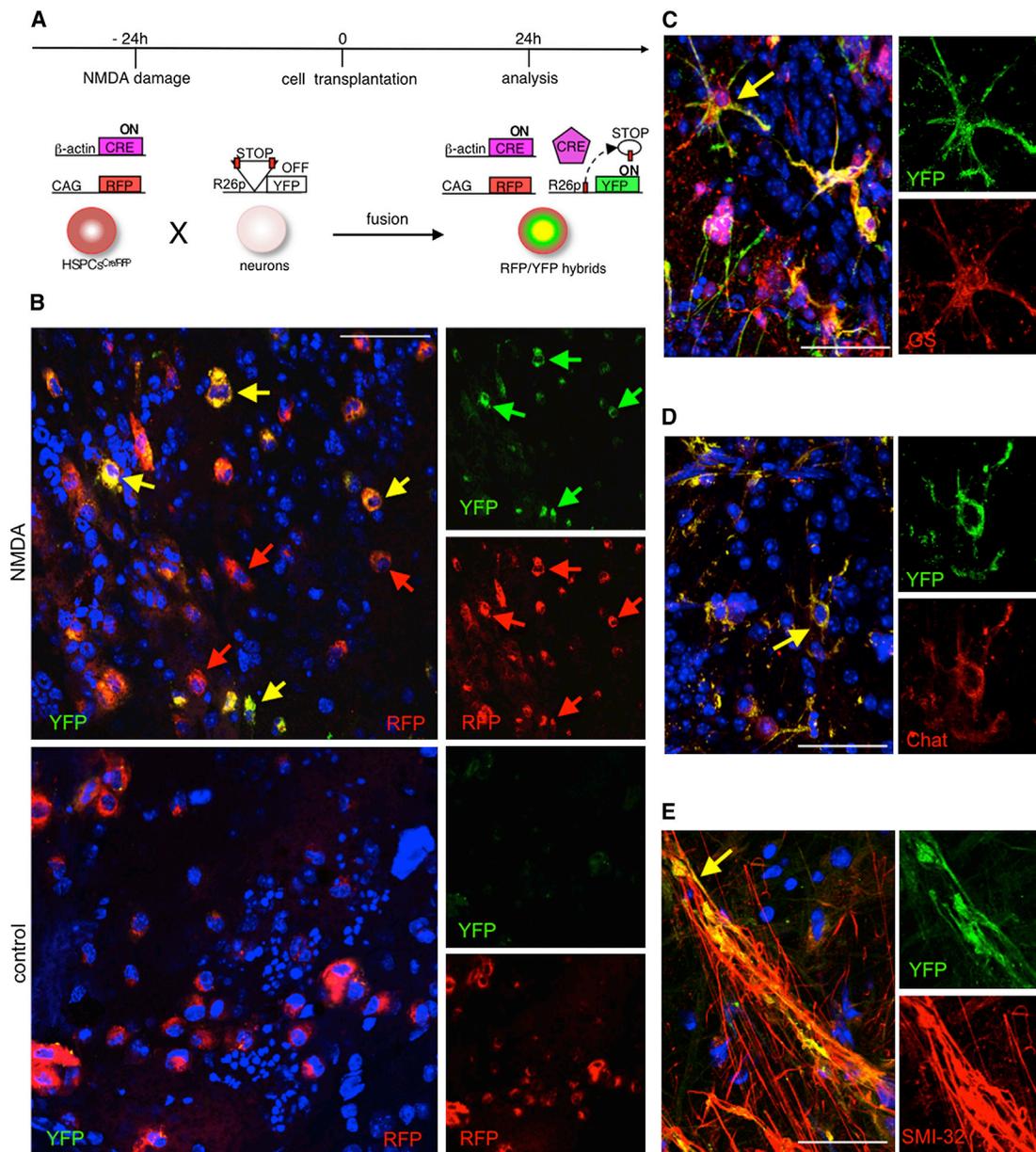
of tissues, although with low efficiency (Alvarez-Dolado et al., 2003; Nygren et al., 2008; Ogle et al., 2005; Vassilopoulos and Russell, 2003; Wang et al., 2003). However, it remains to be seen if retinal damage can enhance fusion efficiency of BM cells and if neurons can thereby be reprogrammed within retinas of adult vertebrates.

Thus, we first investigated whether as a consequence of a retinal injury BM-derived HSPCs can fuse with neurons in the retina, which represents the simplest CNS tissue (Figure S1A). With this aim, we induced retinal damage by injecting NMDA into the vitreous of transgenic mice carrying a LoxP-STOP-LoxP-YFP [R26Y] allele. We left the contralateral eyes undamaged as the controls. NMDA caused apoptosis of the ganglion and amacrine neurons of the retina (Figure S1B), as shown previously (Osakada et al., 2007), but not the incidental expression of yellow fluorescent protein (YFP) (Figure S1C). Then, HSPCs<sup>Cre/RFP</sup> (HSPCs expressing Cre recombinase and red fluorescent protein [RFP]) isolated from CRE-RFP double-transgenic donor mice were transplanted in both R26Y eyes. The retinas were harvested 24 hr after transplantation. In the case of fusion between the injected HSPCs<sup>Cre/RFP</sup> and R26Y retinal neurons, we would expect expression of YFP in hybrid cells due to the stop codon excision by Cre (Figure 1A).

In the damaged eyes, a large number of YFP<sup>+</sup>/RFP<sup>+</sup> hybrids were seen in retinal flat mounts. In contrast, there were no YFP<sup>+</sup> hybrids in the contralateral, nondamaged retinas (Figure 1B). Fusion efficiency between the transplanted HSPCs and retinal cells was evaluated in different ways. We counted the number of hybrids in vertical sections in the area of damage; i.e., in the ganglion cell layer and the inner nuclear layer of the retinal tissue. On average, we obtained 6.5% YFP<sup>+</sup>/RFP<sup>+</sup> cells with respect to the total  $5 \times 10^5$  injected RFP<sup>+</sup> cells (Table S1); however, this percentage was increased to 70% when we evaluated the number of YFP<sup>+</sup>/RFP<sup>+</sup> cells relative to the total RFP<sup>+</sup> cells in the area of damage (Figures S2A and S2B). In addition, fusion was evaluated and confirmed by fluorescence-activated cell sorting (FACS) analysis (Figure S2C). Tetraploid cells, which contained double content of DNA (4C), were also present in high amount, as analyzed by flow cytometry (Figure S2D). Furthermore, some of the hybrids were heterokaryons (Figure S2E). These data demonstrated tissue-damage-induced fusion between HSPCs and retinal cells.

We then asked with which retinal cells the transplanted HSPCs had fused. Thus, we analyzed the expression of different retinal cell markers in YFP<sup>+</sup> hybrids. The hybrids were positive for Müller glia (GS), amacrine (Chat), and ganglion (SMI-32) cell markers (Figures 1C–1E), which indicated that the injected HSPCs fused with these types of retinal cells. In addition, we transplanted DiD-labeled HSPCs<sup>R26Y</sup> (HSPCs expressing the R26Y allele labeled with the red membrane dye DiD) into the damaged eyes of GFAP-Cre and CALR-Cre transgenic mice as lineage-tracing mouse models for Müller and amacrine/ganglion cells, respectively. A significant percentage of DiD<sup>+</sup>/YFP<sup>+</sup> hybrids were seen in both models, further confirming the fusion of Müller glia, amacrine, and ganglion neurons with HSPCs (Figures S2F and S2G).

Finally, we also investigated whether different stem cell types transplanted in the damaged eyes could fuse with retinal



### Figure 1. NMDA Retinal Damage Induces Cell Fusion In Vivo

(A) Schematic representation of the cell fusion experimental plan. In vivo cell fusion between HSPCs<sup>RFP/Cre</sup> and retinal neurons of LoxP-STOP-LoxP-YFP mice (R26Y) leads to excision of a floxed stop codon in the retinal neurons and, in turn, to expression of YFP. The resulting hybrids express both YFP and RFP.

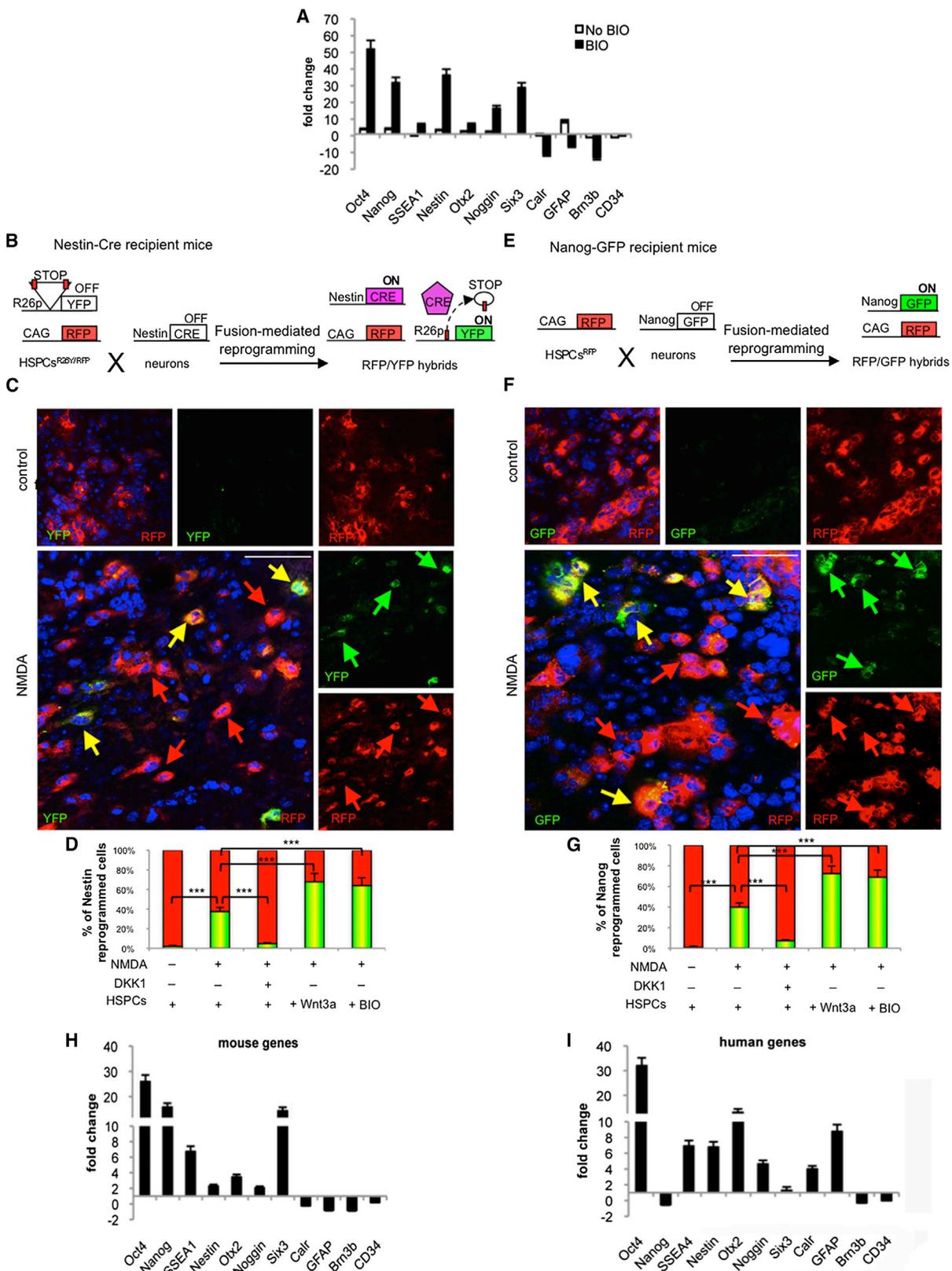
(B) Confocal photomicrographs of flat-mounted retinas focused on the gcl of R26Y NMDA-damaged (NMDA) or healthy (control) eyes of mice transplanted with HSPCs<sup>RFP/Cre</sup> harvested 24 hr after transplantation. Double-positive RFP (red) and YFP (green) hybrids derived from cell fusion are detected in the presence of NMDA damage (NMDA) but not in the undamaged eye (control). Nuclei were counterstained with DAPI (blue). Scale bar: 50  $\mu$ m.

(C–E) Immunohistochemical analysis of the retinal fusion cell partners. YFP<sup>+</sup> hybrids positive for Müller glia (C, GS, red), amacrine (D, Chat, red) or ganglion (E, SMI-32, red) cell markers are detected 24 hr after transplantation of HSPCs<sup>Cre</sup> in NMDA-damaged eyes. Yellow arrows indicate cells positive to both YFP and marker staining. Scale bar: 50  $\mu$ m.

See also Figures S1, S2, and S3.

neurons. Thus, we performed similar experiments by injecting DiD-labeled RSPCs<sup>Cre</sup> isolated from the ciliary margin of Cre transgenic mouse eyes and DiD-labeled ESCs<sup>Cre</sup> into the undamaged and NMDA-damaged eyes of the R26Y mice. Both cell types fused with the retinal cells with good efficiency (Figures

S3A–S3C). Furthermore, we also confirmed that ESCs<sup>Cre</sup> fused with Müller glia, amacrine, and ganglion neurons (Figures S3D and S3E). Overall, our data demonstrate that HSPCs, ESCs, and RSPCs can fuse with retinal neurons in vivo upon cell damage.



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### The Wnt/ $\beta$ -Catenin Signaling Pathway Triggers Reprogramming of Retinal Neurons In Vivo

We previously showed that activation of Wnt signaling triggers cell-fusion-mediated reprogramming of neural precursor cells in culture (Lluis et al., 2008, 2011). With this in mind, we tested whether HSPC/retinal-neuron hybrids were reprogrammed after Wnt/ $\beta$ -catenin pathway activation soon after the injection of the cells. Untreated HSPCs<sup>Cre/RFP</sup> and HSPCs<sup>Cre/RFP</sup> in which the Wnt signaling pathway had been previously activated by the GSK-3 inhibitor 6-bromoindirubin-3'-oxime (BIO) (Figure S4A) were injected into the NMDA-damaged eyes of R26Y mice. Twenty-four hours later, we sorted the hybrids from the retinas by FACS and analyzed the expression of precursor and embryonic genes by real-time PCR.

In the FACS-sorted RFP<sup>+</sup>/YFP<sup>+</sup> BIO hybrids (hybrids formed after transplantation of BIO-HSPCs), Oct4, Nanog, SSEA1 (pluripotency genes), Nestin, Otx2, Noggin, and Six3 (neural precursor genes) were all activated (Figure 2A); expression of these genes was not detectable in the nontransplanted NMDA-injured retinas or in the BIO-treated or untreated HSPCs (Figures S4B and S4C). In contrast, the neuron-specific markers Calr and Brn3b, the Müller glial marker GFAP, and the HSPC-specific gene CD34 were downregulated (Figure 2A). None of the precursor/pluripotency markers were expressed in No-BIO hybrids (hybrids formed after transplantation of untreated HSPCs) (Figure 2A). These data suggested that the BIO hybrids were reprogrammed, as precursor/ pluripotent genes were expressed and differentiation markers were downregulated.

To further demonstrate the activation of an in vivo reprogramming process, we analyzed the reactivation of the Nanog and Nestin promoters in adult retinas. We used two different mouse models as recipients: Nestin-Cre (transgenic mice expressing Cre recombinase gene under the control of Nestin promoter in neural precursors) and Nanog-GFP-Puro mice (transgenic mice expressing GFP-puromycin transgenes under the control of the Nanog promoter in the embryo), which allowed us to investigate reprogramming at the neuronal precursor and the embryonic stages, respectively. Therefore, we transplanted HSPCs<sup>R26Y/RFP</sup> into NMDA-damaged eyes of Nestin-Cre mice. Likewise, we transplanted HSPCs<sup>RFP</sup> into NMDA-damaged eyes of Nanog-GFP mice (Figures 2B and 2E). NMDA was injected intravitreally into one eye of a group of mice, while the contralateral eyes remained undamaged as the controls. Importantly, NMDA alone did not activate the transgene expression (Figure S4D). The retinas were harvested 24 hr after cell transplantation. In the case of

reprogramming of the retinal neurons, in these mouse models, we would expect to find double-red/green-positive cells (Figures 2B and 2E).

In both mouse models, in flat-mounted retinas, we found a high number of green<sup>+</sup>/red<sup>+</sup> hybrids, which indicated the reactivation of both Nanog and Nestin promoters (Figures 2C and 2F). To evaluate the efficiency of this in vivo reprogramming, we counted the green-positive reprogrammed cells relative to the total injected red-HSPCs and relative to the red-HSPCs detected in the ganglion cell layer and the inner nuclear layer of the retinal tissue. A percentage of red cells were also green when HSPCs were injected into the NMDA-damaged eyes of Nestin-CRE and Nanog-GFP mice, respectively (Figures 2D–2G; Figures S4E and S4F; Tables S2 and S3), indicating that in the area of damage up to 30% of the hybrids were indeed reprogrammed, as they had reactivated either Nestin or Nanog promoters in the neurons.

NMDA has been shown to activate the Wnt pathway in the retina (Osakada et al., 2007); therefore, to assess the role of activation of the Wnt/ $\beta$ -catenin signaling pathway in the reprogramming of retinal neurons in both of these mouse models, we also injected DKK1 immediately after NMDA injection. DKK1 is an inhibitor of the Wnt pathway (Niida et al., 2004). HSPCs were transplanted after 24 hr, and mice were sacrificed 24 hr later. The amount of double-red<sup>+</sup>/green<sup>+</sup>-reprogrammed hybrids was strongly reduced when DKK1 was administered (Figures 2D and 2G; Figures S4E and S4F; Tables S2 and S3). These results demonstrated that endogenous and damage-dependent activation of the Wnt/ $\beta$ -catenin pathway triggers reprogramming of retinal neurons after their fusion with HSPCs.

Next, we wanted to analyze whether reprogramming of retinal neurons was increased after transplantation of HSPCs in which the Wnt signaling pathway had been previously activated using BIO or by Wnt3a treatment before injection. Surprisingly, 24 hr after transplantation of BIO-pretreated or Wnt3a-pretreated HSPCs in NMDA-damaged eyes of the Nestin-Cre and Nanog-GFP mice, there was a striking increase in the number of reprogrammed (green<sup>+</sup>) hybrids with respect to those seen in NMDA-damaged eyes that received untreated HSPCs (Figures 2D and 2G; Figures S4E and S4F; Tables S2 and S3). Increased reprogramming after transplantation of BIO-treated HSPCs was also evaluated and confirmed by FACS analysis (Figure S4G).

Of note, no reprogramming events were seen after the injection of BIO-treated HSPCs into nondamaged eyes (Figure S4H), because there was no hybrid formation (Figure S7F, compare

#### Figure 2. Activation of the Wnt Signaling Pathway Enhances Neuron Reprogramming after HSPC Fusion In Vivo

(A) Gene expression profile of fluorescence-activated cell sorted RFP<sup>+</sup>/YFP<sup>+</sup> hybrids after transplantation of untreated (No BIO, white bars) and BIO-treated (black bars) HSPCs<sup>Cre/RFP</sup> in R26Y NMDA-damaged eyes. Data are real-time PCR quantifications plotted as the fold change with respect to controls from complementary DNAs of NMDA-damaged retinas mixed with either untreated HSPCs or BIO-treated HSPCs. Data are mean  $\pm$ SEM (n = 6).

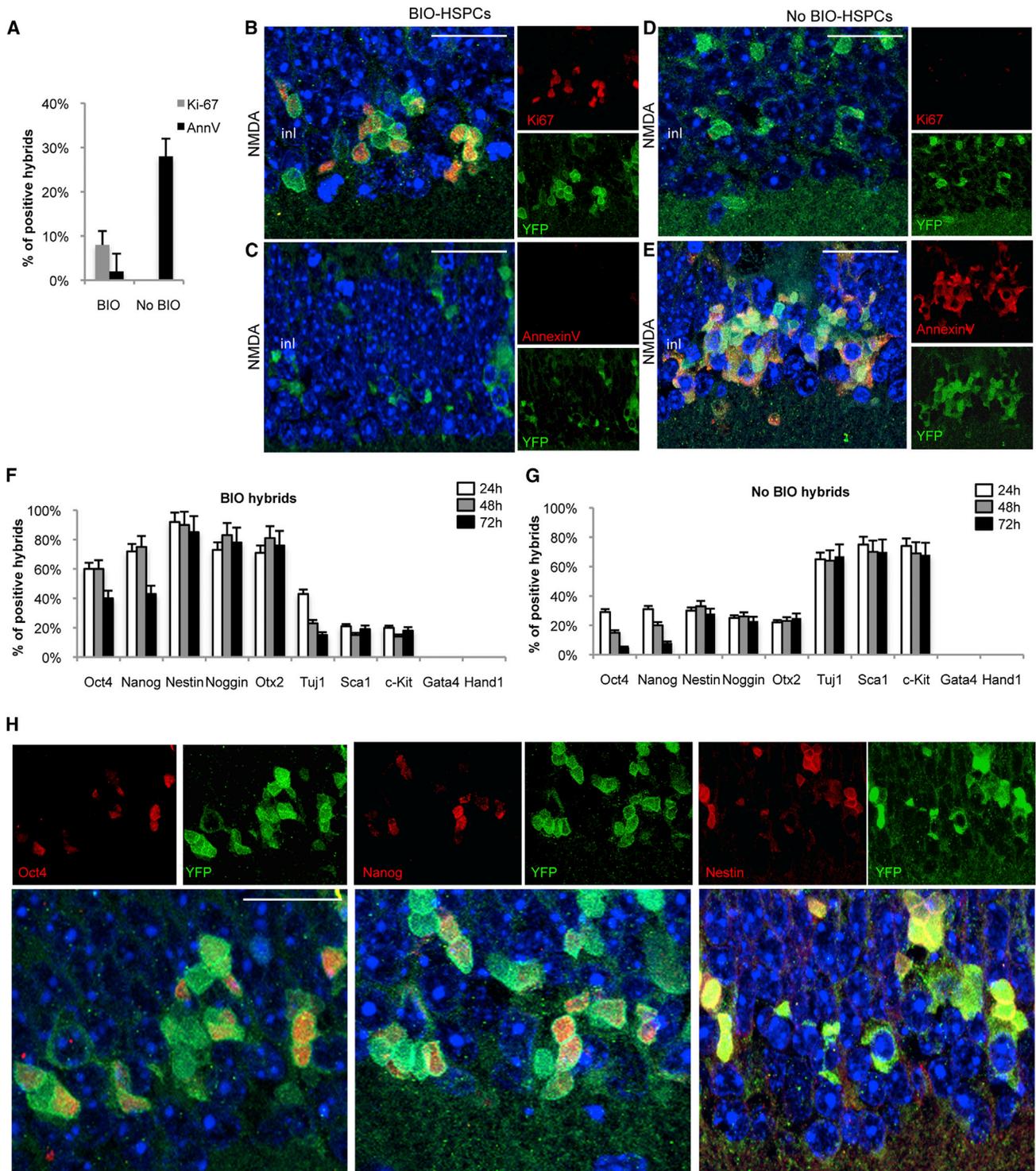
(B and E) Schemes of cell-fusion-mediated reprogramming to identify Nestin (B) or Nanog (E) promoter activation in transgenic mice.

(C and F) Double-red<sup>+</sup>/green<sup>+</sup>-reprogrammed hybrids were detected only in the presence of NMDA damage in flat-mounted retinas after transplantation of HSPCs<sup>R26Y/RFP</sup> in Nestin-Cre (C) and HSPCs<sup>RFP</sup> in Nanog-GFP (F) eyes. Scale bars: 50  $\mu$ m.

(D and G) Quantification of double-red<sup>+</sup>/green<sup>+</sup> hybrids activating Nestin (D) and Nanog (G) promoters evaluated according to Wnt signaling modulation. Undamaged or NMDA-damaged retinas treated or not with DKK1 were transplanted after 24 hr with either untreated, Wnt3a-treated (Wnt3a), or BIO-treated (BIO) HSPCs. Data are mean  $\pm$ SEM (n = 90) counted in vertical sections (as for Figure S2B). \*\*\*p < 0.001.

(H and I) Mouse (H) and human (I) gene expression profiles of fluorescence-activated cell sorted DiD<sup>+</sup>/GFP<sup>+</sup> hybrids after transplantation of DiD-labeled human CD34<sup>+</sup> HSPCs in NMDA-damaged eyes of Nanog-GFP mice. Data are mean  $\pm$ SEM (n = 9).

See also Figures S4, S5, S6, S7, and S8.



**Figure 3. Reprogrammed Hybrids Can Proliferate and Differentiate**

(A) Quantification of mitotic (Ki-67<sup>+</sup>) and apoptotic (Annexin V<sup>+</sup>) YFP<sup>+</sup> hybrids after transplantation of untreated (No BIO) or BIO-treated (BIO) HSPCs<sup>Cre</sup> in NMDA-damaged R26Y eyes. Data are mean ± SEM; n = 30. \*\*\*p < 0.001.

(B–E) Ki67 (B and D, red) and Annexin V (C and E, red) staining for YFP<sup>+</sup> (green)-reprogrammed hybrids obtained 24 hr after injection of BIO-treated (BIO-HSPCs) or untreated-HSPCs<sup>Cre</sup> in NMDA-damaged R26Y eyes. Nuclei were counterstained with DAPI (blue).

(F and G) Quantification of YFP<sup>+</sup> hybrids positive to different markers, as counted after immunostaining of sections (as for Figure S2B) at different times after transplantation of BIO-treated (F) and untreated (G) HSPCs<sup>Cre</sup> into NMDA-damaged eyes of R26Y mice. Data are mean ± SEM (n = 30).

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lanes 2 and 4, gray bars), confirming that the tissue damage is necessary for cell-fusion-mediated retinal neuron reprogramming. All of these data clearly indicate that activation of Wnt signaling induces a cell-fusion-mediated reprogramming process *in vivo*.

To further confirm the role of  $\beta$ -catenin during *in vivo* cell reprogramming upon Wnt signaling activation, in damaged retinas of Nanog-GFP mice, we transplanted HSPCs isolated from a strain carrying the  $\beta$ -catenin gene flanked by Flox sites. DiD-labeled HSPCs  $\beta\text{cat}^{+/+}$  were treated with tamoxifen to delete  $\beta$ -catenin and further left untreated or treated with BIO (Figures S5A and S5C). Very few reprogramming events were seen after injection of either the untreated or the BIO-treated HSPCs  $\beta\text{cat}^{\text{flox}/\text{flox}}$  (Figures S5B and S5D). These results show that stabilization of  $\beta$ -catenin in the transplanted HSPCs is essential to allow *in vivo* reprogramming process after cell fusion.

Finally, we investigated whether both the retinal neuron genome and the transplanted HSPC genome underwent reprogramming after fusion. Thus, we transplanted DiD-labeled human CD34<sup>+</sup> HSPCs (hHSPCs) treated with BIO into the damaged eyes of Nanog-GFP mice to obtain interspecies hybrids (Figures S6A–S6D). In fluorescence-activated cell sorted DiD<sup>+</sup>/GFP<sup>+</sup> hybrids, there was activation of mouse pluripotent genes, such as Oct4, Nanog and SSEA1, and of neuronal precursor genes, such as Nestin, Otx2, Noggin, and Six3; however, the neuronal-lineage-specific genes Calr and Brn3b and the activated Müller glia marker GFAP were downregulated (Figure 2H). Also, the hHSPC genome underwent reprogramming as some pluripotency/precursor genes (such as hOct4, hSSEA1, hNestin, hOtx2, and hNoggin) were activated, while the HSPC marker hCD34 was downregulated (Figure 2I). These results suggested that a Wnt-dependent reprogramming process of both genomes is activated in the hybrids. In addition, lineage genes (such as hCalr and hGFAP) were activated in the human genome (Figure 2I), indicating that HSPC nuclei can be reprogrammed and differentiated toward a neuronal lineage.

Given that it was surprising to observe reprogramming at the embryonic stage after fusion of HSPCs with neurons, we also investigated activation of the Nanog-GFP transgene after transplantation of ESCs and RSPCs into NMDA-injured eyes. As expected, in the case of the ESC transplantation, we observed reprogramming of the retinal neurons, which was also dependent on activation of the Wnt signaling pathway. DKK1 blocked reprogramming, while GFP-positive cells were strikingly increased when ESCs were pretreated with BIO or Wnt3a before being transplanted (Figures S7A–S7C; Table S4).

To further confirm pluripotency of ESC/retinal-neuron hybrids, we performed clonal analysis *in vitro*. NMDA-damaged retinas of Nanog-GFP-Puro mice were transplanted with untreated or BIO-treated ESCs expressing RFP. Transplanted nondamaged retinas were used as control. Retinas harvested 24 hr after transplantation were disaggregated and the cells were cultured in

ESC medium under puromycin selection. The cells formed reprogrammed clones, as they were puromycin resistant and GFP<sup>+</sup>, indicating activation of the transgene Nanog promoter (Figures S8A and S8B). In addition, they were positive for alkaline phosphatase (AP) staining (Figure S8C) and expressed Oct4 and Nanog (Figure S8E). A high number of reprogrammed colonies were selected only from retinas transplanted with BIO-treated ESCs (Figure S8D). The reprogrammed clones were hybrids, as they expressed both GFP and RFP (Figures S8A and S8B). Furthermore they were tetraploid, as 50% of the GFP<sup>+</sup> RFP<sup>+</sup> cells contained double DNA content (4n), as evaluated by FACS analysis (Figures S8F and S8G).

We also transplanted BIO-treated ESC<sup>Cre</sup> in R26Y-damaged eyes; YFP<sup>+</sup> teratomas were seen in mice sacrificed 1 month after transplantation (Figure S7D), which confirmed that reprogrammed hybrids could differentiate in different lineages. All these results showed that the hybrids formed after fusion of BIO-treated ESCs are reprogrammed and pluripotent. In contrast, we observed no reprogramming events after injection of RSPCs into the NMDA-injured eyes of the Nanog-GFP mice, even in the case of BIO pretreatment of transplanted RSPCs (Figure S7E).

Finally, we also ruled out an effect of BIO in the enhancement of fusion events *in vivo*. BIO pretreatment did not enhance the fusion efficiency of HSPCs, ESCs, or RSPCs injected into the NMDA-damaged eyes of the R26Y mice (Figure S7F).

In conclusion, here we have shown that activation of the Wnt/ $\beta$ -catenin signaling pathway triggers the reprogramming of retinal neurons back to an embryonic/neuronal-precursor stage and that this occurs following damage-dependent cell-cell fusion of HSPCs and ESCs but not of RSPCs.

### Reprogrammed Hybrids Can Proliferate and Differentiate *In Vivo*

Next, we investigated the proliferative potential of the newly generated hybrids. We injected BIO-treated and untreated HSPCs<sup>Cre</sup> into the NMDA-damaged eyes of a group of R26Y mice and retinal sections were analyzed 24 hr later.

BIO treatment led to the formation of mitotic hybrids that re-entered the cell cycle, as a percentage of the YFP<sup>+</sup> cells were also Ki67<sup>+</sup> and BrdU<sup>+</sup> (Figures 3A and 3B; Figure S9A); moreover, only a few YFP<sup>+</sup> cells were Annexin V<sup>+</sup> and therefore committed to an apoptotic fate (Figures 3A and 3C). The higher percentage of BrdU<sup>+</sup> hybrids with respect to the Ki67<sup>+</sup> hybrids suggested that the majority of these replicated their DNA and entered into G0, probably after their differentiation. In contrast, no proliferation was seen in hybrids formed after injection of untreated HSPCs, where a high percentage of the hybrids underwent apoptosis (No BIO; Figures 3A, 3D, and 3E; Figure S9A). Similar results were obtained after transplantation of BIO-treated or untreated (No BIO) ESCs (Figure S9B).

Overall, our results show that upon activation of the Wnt/ $\beta$ -catenin pathway in the transplanted cells, the newly reprogrammed hybrids can proliferate through re-entering into the

(H) YFP<sup>+</sup> hybrids (green) obtained 24 hr after transplantation of BIO-treated HSPCs<sup>Cre</sup> in NMDA-damaged R26Y eyes stained with Oct4, Nanog, and Nestin antibodies (red). Nuclei were counterstained with DAPI.

Scale bars: 50  $\mu\text{m}$  (B, C, D, E, and H).

See also Figure S9.

cell cycle. In contrast, when Wnt signaling is not activated, the fate of the hybrids is to embark into apoptosis.

We then analyzed the *in vivo* differentiation potential of the reprogrammed retinal neurons of R26Y injected with BIO-treated and untreated HSPCs<sup>Cre</sup>. The mice were sacrificed 24, 48, and 72 hr after transplantation. The percentages of YFP<sup>+</sup> hybrids also positive for each marker were determined from retinal sections. There were many Oct4-expressing and Nanog-expressing hybrids 24 and 48 hr after transplantation of BIO-HSPCs, while these decreased by 72 hr (Figures 3F and 3H). At 24 hr, YFP<sup>+</sup> hybrids were already expressing Nestin, Noggin, and Otx2 (neuroectoderm), and this expression was maintained at the subsequent times analyzed (48 and 72 hr) (Figures 3F and 3H). Conversely, the neuronal terminal differentiation marker Tuj-1 and the HSPC markers Sca1 and c-Kit were silenced (Figure 3F).

On the other hand, after fusion with untreated HSPCs, a low number of YFP<sup>+</sup> hybrids reactivated Oct4 and Nanog, although only at 24 hr. These also activated Nestin, Noggin, and Otx2 at low levels. Instead, the majority of these hybrids maintained constant expression of Tuj-1, Sca-1, and c-Kit (Figure 3G). Overall, this indicates activation of a less efficient reprogramming process. Both in the absence and presence of BIO treatment, the mesoderm marker Gata4 and the endoderm marker Hand1 were never expressed (Figures 3F and 3G).

In contrast, after injection of BIO-treated ESCs<sup>Cre</sup> in the damaged R26Y retinas, a delay in the neuroectodermal differentiation potential of the resulting hybrids (as Nestin, Noggin, and Otx2 were expressed more at 72 hr, while Oct4 and Nanog were highly expressed from 24 hr to 72 hr) was seen. Furthermore, the hybrids also expressed Gata4 and Hand1 (Figure S9C). Of note, Oct4 and Nanog were poorly expressed in the hybrids formed after fusion of untreated ESCs<sup>Cre</sup>, while Tuj-1 expression was maintained, which indicates activation of a poor reprogramming process (Figure S9D).

Thus, we concluded that the BIO-ESC-derived hybrids were pluripotent, as they can differentiate in mesoderm, endoderm, and neuroectoderm lineages; in contrast, the BIO-HSPC-derived hybrids were transiently reprogrammed back to an embryonic-like state and rapidly embarked into neuroectoderm differentiation exclusively.

### Reprogrammed Hybrids Can Regenerate the Injured Retina

Having seen that the reprogrammed hybrids can proliferate and differentiate toward neuroectodermal lineage, we aimed to evaluate their long-term differentiation and regenerative potential. HSPCs<sup>Cre</sup> were pretreated with BIO to activate Wnt signaling and transplanted into the NMDA-damaged R26Y eyes; untreated HSPCs<sup>Cre</sup> were transplanted as the control. The mice were sacrificed 4 weeks later (Figure 4A). Analysis of flat-mounted retinas transplanted with BIO-HSPCs<sup>Cre</sup> showed a high number of YFP<sup>+</sup> hybrids (Figure 4B) that were positive for expression of ganglion (SMI-32) and amacrine (Chat) neuron markers (Figure 4C).

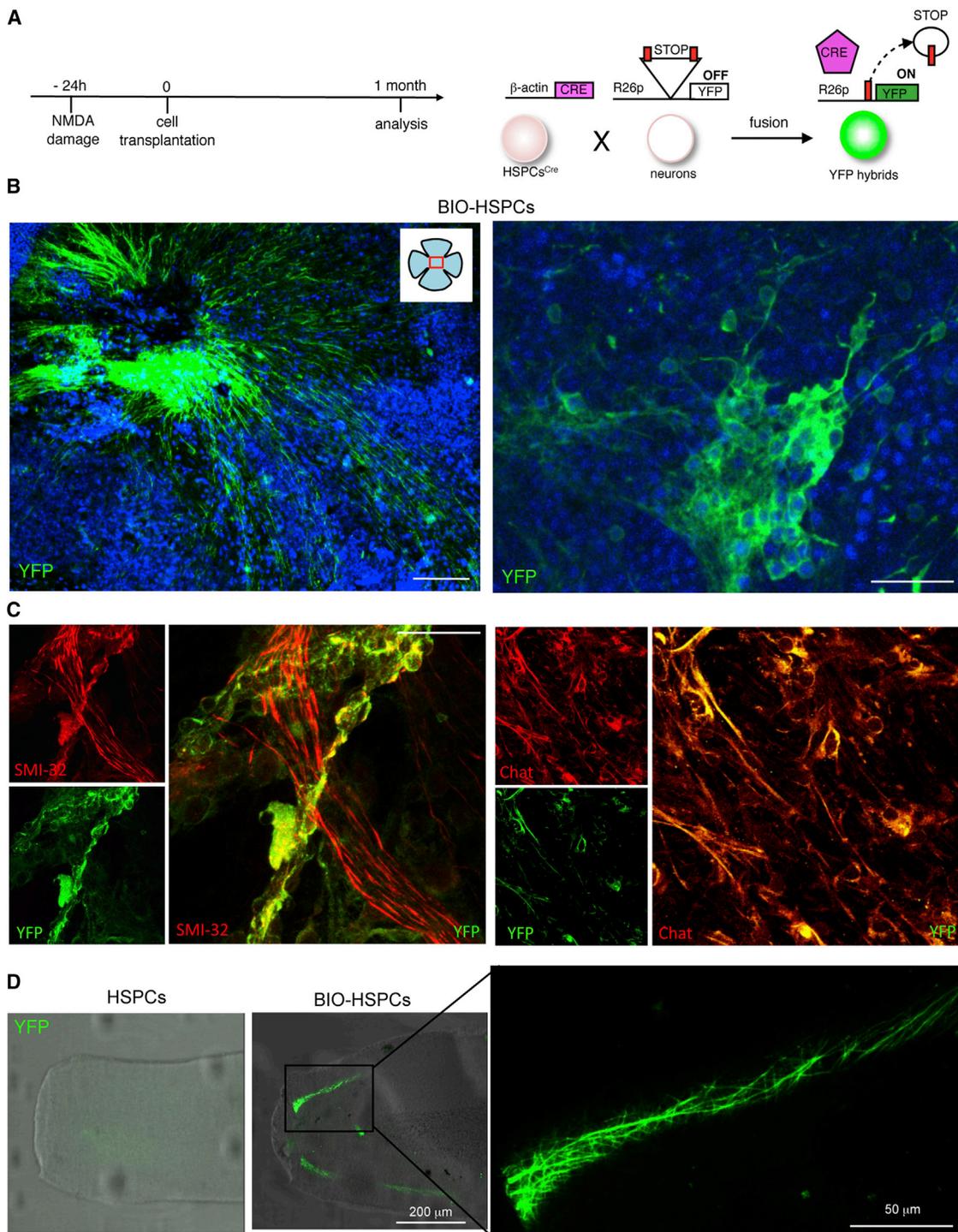
We then analyzed the optical nerves 24 hr and 1 month after transplantation. Remarkably, we observed YFP<sup>+</sup> axons in the 1-month optical nerves, possibly derived from projections of the regenerated ganglion neurons (Figures 4B–4D). In contrast,

retinas transplanted with untreated HSPCs<sup>Cre</sup> showed very few YFP<sup>+</sup> hybrids (Figure S10A) with no YFP<sup>+</sup> axons along the optical nerves (Figure 4D, untreated HSPCs). Interestingly, no YFP<sup>+</sup> axons were seen in the optical nerves 24 hr after transplantation of BIO-HSPCs (Figure S10B), which indicates that the newly generated ganglion neurons project their axons a while after transplantation, during the regeneration process (Figure 4D).

NMDA treatment induces recruitment of macrophages in the eye (Sasahara et al., 2008). Indeed, as expected, in retinas harvested 24 hr after transplantation, a number of the YFP<sup>+</sup> hybrids were positive to the monocyte/macrophage markers CD45 and Mac1, which suggested phagocytosis of some transplanted HSPCs<sup>Cre/RFP</sup> by endogenous macrophages carrying the R26Y allele or phagocytosis of some YFP<sup>+</sup> hybrids themselves (Figures S10C, S10D, and S10G). Interestingly, these YFP<sup>+</sup> hybrids positive to monocyte/macrophage markers were drastically decreased in retinas harvested 1 month after transplantation (Figures S10E–S10G). This indicates that some hybrids can be phagocytosed soon after transplantation but there remain a sufficient number of hybrids that can regenerate the retinas.

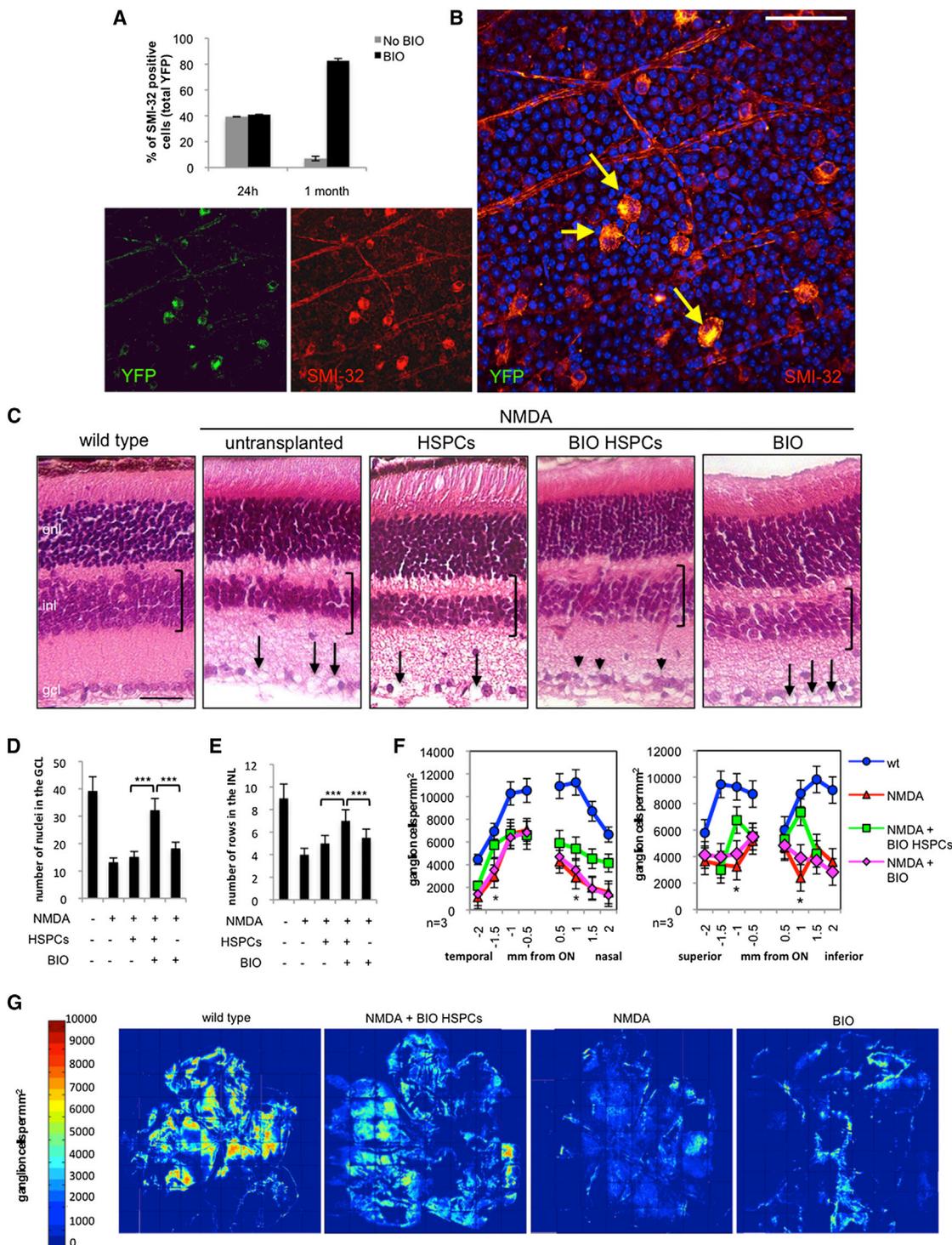
To evaluate the levels of surviving hybrids that regenerate the retinas, we used FACS analysis to count the YFP<sup>+</sup> cells over all retinal cells both 24 hr after and 1 month after transplantation of the BIO-treated HSPCs<sup>Cre</sup> in damaged R26Y eyes. We found up to a 4- to 5-fold increase in the YFP<sup>+</sup> cells 1 month after transplantation, which indicated newly generated cells (Figure S10H). Next, in flat-mounted retinas, we counted the number of YFP<sup>+</sup> cells that were positive for the ganglion marker SMI-32. Interestingly, 80% of the YFP<sup>+</sup> neurons expressed SMI-32 1 month after transplantation of the BIO-treated HSPCs, which indicated regeneration of the retina by differentiation of the hybrids (Figures 5A and 5B). In addition, expression of the ganglion markers Calr and Brn3b in the YFP-sorted hybrids was comparable to the wild-type retina 1 month after transplantation of the BIO-treated HSPCs (Figure S10I). In contrast, with transplantation of the untreated HSPCs, there was a drastic drop in the number of SMI-32<sup>+</sup> cells and very little expression of Calr and Brn3b 1 month after transplantation (Figure 5A; Figure S10I). Of note, and in agreement with previous findings (Otani et al., 2004), HSPC-transplanted retinas expressed retinal pigment epithelium, indicating a possible transdifferentiation of the untreated HSPCs toward the epithelial lineage (Figure S10J).

Next, we analyzed the occurrence of neuron regeneration by histological analysis of retinal vertical sections. Interestingly, in retinas transplanted with BIO-HSPCs, the number of nuclei in the ganglion cell layer (Figures 5C and 5D, gcl) and the number of nuclear rows in the inner nuclear layer (Figures 5C and 5E, inl) were comparable to wild-type retinas and substantially increased with respect to nontransplanted retinas and retinas transplanted with untreated HSPCs (Figures 5C–5E). This indicates retinal regeneration. We also investigated the nuclear density of the ganglion neurons in the flat-mounted retinas by counting the total number of ganglion nuclei in the whole retinas harvested 1 month after transplantation. Remarkably, there was a significant increase in the number of nuclei in BIO-HSPCs<sup>Cre</sup>-transplanted retinas, with respect to the nontransplanted retinas (Figure 5F). However, newly generated ganglion neurons were not uniformly distributed, as shown by the nuclear density



**Figure 4. Long-Term Differentiation Potential of Hybrids Obtained after Cell-Fusion-Mediated Reprogramming**

(A) Experimental strategy used to identify YFP<sup>+</sup> hybrids 1 month after BIO-treated or untreated HSPCs<sup>Cre</sup> in NMDA-damaged R26Y retinas. (B) YFP<sup>+</sup> neurons were detected in NMDA-injured R26Y retinal flat mounts 1 month after BIO-HSPCs<sup>Cre</sup> transplantation. Nuclei were counterstained with DAPI (blue). Right: higher magnification of the YFP<sup>+</sup> neurons. (C) YFP<sup>+</sup> differentiated hybrids (green) expressing either the ganglion cell marker SMI-32 (left, red) or the amacrine cell marker Chat (right, red). (D) YFP<sup>+</sup> axons (green) detected in optic nerves from eyes transplanted with BIO-HSPCs<sup>Cre</sup> but not with untreated HSPCs<sup>Cre</sup>. Right: higher magnification of the YFP<sup>+</sup> axons (green) in the optic nerve. Scale bars: 50  $\mu$ m (B, C, and D) or 200  $\mu$ m (D). See also Figure S10.



**Figure 5. NMDA-Damaged Retinas Can Be Regenerated after Fusion of Transplanted BIO-Treated HSPCs**

(A and B) Evaluation of the percentage of YFP<sup>+</sup> (green) hybrids also positive to SMI-32 staining (red) in flat-mounted retinas harvested 24 hr or 1 month after transplantation of BIO-treated (BIO, black bars) or untreated (No BIO, gray bars) HSPCs<sup>Cre</sup> in NMDA-damaged R26Y eyes (n = 3). Twenty different random fields were analyzed for each retina. A representative image of regenerated ganglion cells (yellow arrows) double positive for YFP (green) and SMI-32 (red) is shown in (B). Scale bar: 50  $\mu$ m.

(C) Hematoxylin and eosin staining showing increases in thickness of the inner nuclear layer (inl, square brackets) and regeneration of the ganglion cell layer (gcl, arrowheads) in NMDA-damaged retina 1 month after BIO-HSPCs<sup>Cre</sup> transplantation. Transplantation of untreated HSPCs<sup>Cre</sup> or BIO alone does not induce regeneration. Arrows indicate ganglion cell loss in the NMDA-damaged retinas. Arrowheads indicate ganglion cell nuclei. Scale bars: 50  $\mu$ m.

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maps, which indicated nonhomogenous retinal regeneration (Figure 5G).

Finally, we also tested whether the injection of BIO and of Wnt3a without cell transplantation in damaged retinas can trigger regeneration. We did not find substantial increases in the numbers of nuclei in the ganglion cell layer and of the nuclear rows in the inner nuclear layer, and we did not observe increased numbers of nuclei in the whole flat-mounted retinas (Figures 5C–5G; Figures S10K and S10L). These data demonstrate that only if Wnt signaling is activated in the transplanted HSPCs can partial regeneration of the damaged retinal cells be achieved after fusion of transplanted cells.

As we observed regeneration only after transplantation of BIO-treated HSPCs, we investigated functional rescue in these regenerated retinas. For this, a group of mice received NMDA and were either nontransplanted or transplanted with BIO-treated HSPCs. To test the responses of the ganglion neurons, we used a multielectrode array that enables simultaneous extracellular recordings from many retinal ganglion cells. Although there was some variability across different mice, the proportion of clear light-driven retinal ganglion cells was significantly lower in damaged retinas than in the control retinas (Figure 6A). In contrast, the retinas transplanted with BIO-treated HSPCs showed much higher proportions of ganglion neurons with light-evoked responses with respect to the NMDA retinas, which were comparable with the control retinas (Figure 6A).

After uniform flash stimulus, retinal ganglion cells can respond with an increase in firing rate to the onset of the flash (ON cells), to the offset (OFF cells), to onset and offset (ON-OFF cells) and with a firing rate transiently decreased at the onset (SUPPRESSED cells) (Nirenberg and Meister, 1997). Representative examples of sorted spikes waveforms, raster plots and peristimulus time histograms of the ganglion cells of control, nontransplanted, and BIO-HSPC-transplanted retinas are shown (Figure 6B). Interestingly, BIO-HSPC-transplanted retinas showed usually spikes of higher amplitude than NMDA nontransplanted retinas.

Since more than half of the retinal ganglion cells recorded in all the retinas were ON cells, we examined the response to the light onset in these cells. ON cells had longer latencies in damaged retinas than in the control and BIO-treated HSPC transplanted retinas ( $p < 0.01$ ). The mean latencies to the ON peak were as follows (mean  $\pm$ SD): control,  $177.9 \pm 29.4$  ms; NMDA alone,  $234.6 \pm 49.1$ ; and NMDA + BIO-HSPCs,  $193.6 \pm 56.9$ . The smallest observation (sample minimum), lower quartile, median, upper quartile, and largest observation (sample maximum) of each sampled group are shown in Figure 6C.

Finally, to study the ganglion cell responses to changing light intensities, the retinas were randomly stimulated with full-field flashes with ten different light intensities ranging from black ( $0.46 \text{ cd/m}^2$ ) to white ( $196.3 \text{ cd/m}^2$ ). Our results show that a lower

percentage of cells in NMDA-damaged retinas respond to medium-intensity gray level (corresponding to  $35.5 \text{ cd/m}^2$ ) with respect to control and BIO-HSPC-transplanted retinas (Figure 6D).

### Endogenous BMC Fusion-Mediated Reprogramming of Retinal Neurons

BMCs are recruited into the eyes upon damage (Sasahara et al., 2008). We thus investigated whether Wnt-dependent reprogramming of retinal neurons upon fusion with BMCs can occur as an endogenous process after NMDA damage. We generated chimeric mice by replacing the BM of sublethally irradiated R26Y recipient mice with the BM of donor RFP-Cre mice (Figure 7A). The repopulation of the BM with cells of donor origin was analyzed (Table S5). NMDA damage was induced in one eye of each group of chimeric mice, and then 24 hr later the mice were sacrificed (Figure 7A). Interestingly, after NMDA damage, some RFP<sup>+</sup> BM-derived cells were also YFP<sup>+</sup>, indicating fusion of endogenous BMCs recruited into the eyes (Figures 7B and 7C; Figure S11A; Table S6). Various YFP<sup>+</sup> hybrids were also positive for Sca1, Chat, SMI-32, and GS (Figure S11B), which suggests that endogenous BMCs can also fuse with ganglion, amacrine, and Müller cells upon damage. No YFP<sup>+</sup>/RFP<sup>+</sup> cells were found in undamaged retinas (Figure 7B).

Next, we investigated whether reprogramming can occur after BMC recruitment and fusion with retinal neurons. Here, we transplanted BMCs<sup>R26Y</sup> into a group of sublethally irradiated Nestin-Cre mice to generate chimeric mice (Figure 7D). The reactivation of the Nestin-Cre transgene and the consequent YFP expression enabled us to identify reprogramming events after BMC recruitment in the eye.

One month after BM replacement, the injection of BIO into NMDA-damaged eyes of the chimeric mice led to the formation of YFP<sup>+</sup> hybrids, which indicated Nestin promoter reactivation (Figure 7E). In control eyes, which were not treated with BIO, no YFP<sup>+</sup> hybrids were seen (Figure 7E). Some YFP<sup>+</sup> hybrids were in active mitosis (Figure 7F, Ki67<sup>+</sup>), and only a few were dying (Figure 7F, Annexin V<sup>+</sup>); moreover, some of the hybrids were positive for Oct4 and Nanog staining, which suggested reprogramming of retinal neurons after their fusion with recruited BMCs (Figure 7G; Figure S11C; Table S6). In conclusion, after recruitment of BMCs, activation of fusion-mediated reprogramming of retinal neurons can occur in the eye if the Wnt pathway is activated.

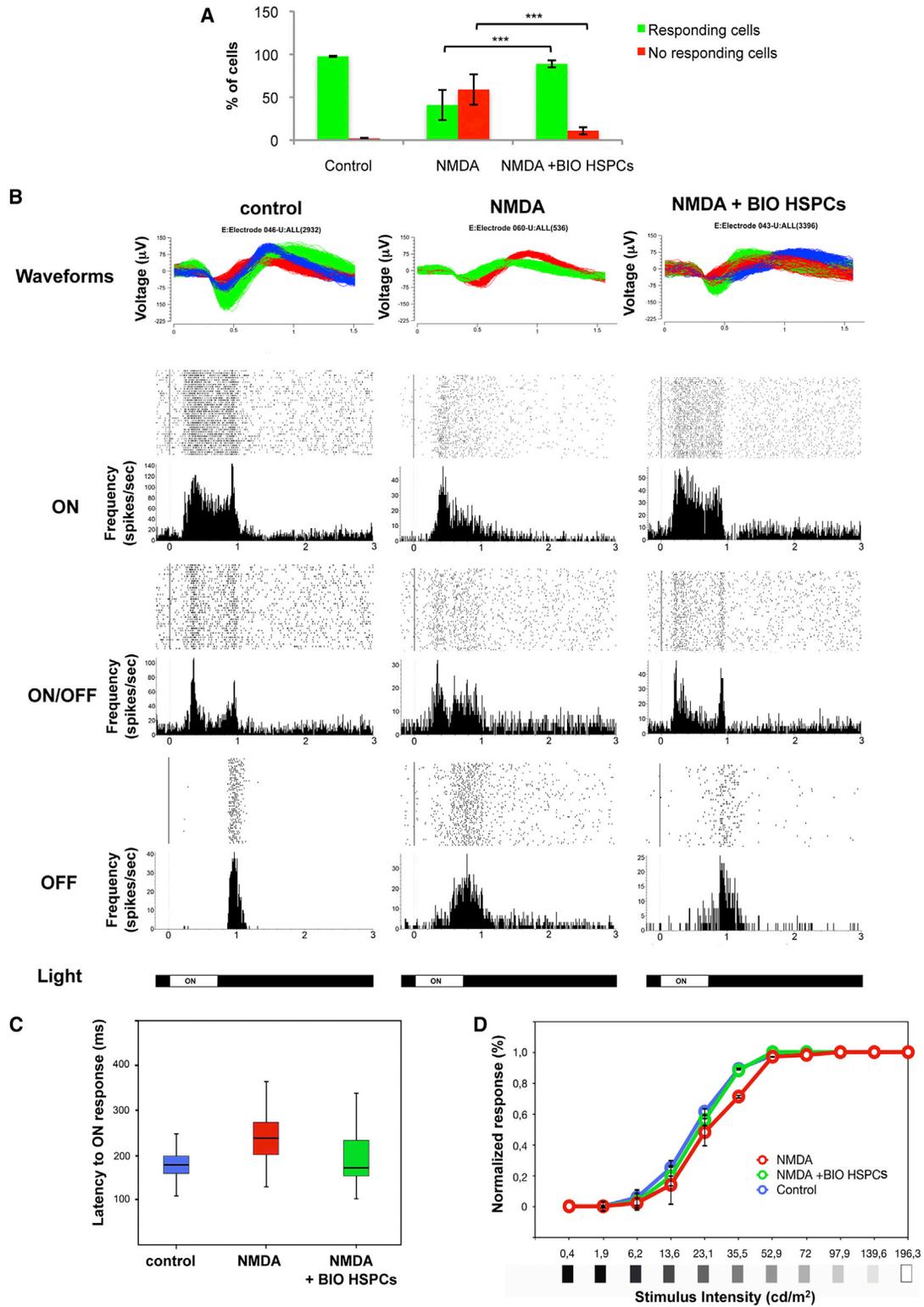
### DISCUSSION

Here, we have demonstrated that the canonical Wnt/ $\beta$ -catenin signaling pathway mediates the reprogramming of retinal neurons in vivo. In addition, we have shown that spontaneous cell

(D and E) Quantification of ganglion nuclei in the ganglion cell layer (D, gcl) and nuclear rows in the inner nuclear layer (E, inl) as counted in vertical retinal sections of undamaged or NMDA-damaged retinas treated as in (C). Data are mean  $\pm$ SEM ( $n = 30$ ). \*\*\* $p < 0.001$ .

(F) All the nuclei excluding those of endothelial cells were counted in the ganglion cell layer along the nasotemporal (left) and dorsoventral (right) axes and plotted as cells per  $\text{mm}^2$ . A total of 80 different images composing the whole retina were counted for each sample. Data are mean  $\pm$ SEM ( $n = 3$ ). \* $p < 0.01$  between damaged retinas transplanted with BIO-HSPCs and untransplanted damaged retinas. ON, optic nerve.

(G) Total cells in the ganglion cell layer, excluding endothelial cells, counted as in (F), were plotted as density maps, as indicated in the color key. See also Figure S10.



**Figure 6. Multielectrode Recordings from Flat-Mounted Mouse Retinas**

(A) Percentage of neurons responding or not responding to light in control retinas, NMDA-damaged retinas, and NMDA-damaged retinas transplanted with BIO-HSPCs. Data are mean  $\pm$ SEM (n = 5 for each group). \*\*\*p < 0.001.

(B) Representative examples of sorted spikes waveforms (top graphs; each color represents a single sorted unit), raster plots of spiking responses, and peristimulus time histograms (PSTHs, bin = 10 ms) (bottom graphs) from typical retinal ganglion cells of control, NMDA-damaged, and NMDA + BIO-HSPC retinas.

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fusion can occur in the mouse retina after injury and that a proportion of fusion hybrids proliferate if they are reprogrammed by Wnt activity. Furthermore, we have also shown that if the hybrids are not reprogrammed, they undergo apoptosis. Surprisingly, the reprogrammed hybrids can regenerate the damaged retinal tissue, although the regeneration is not homogenous. Functional rescue of ganglion neurons is also observed in regenerated retinas. Finally, we have shown that after exogenous activation of the Wnt signaling pathway in the eye, BM-derived cells that are recruited into the injured retina can fuse and reprogram the retinal neurons.

Adult SPCs can contribute to a wide spectrum of differentiated cells. Transplanted BMCs can fuse and acquire the identity of liver cells, Purkinje neurons, kidney cells, and epithelial cells. This plasticity has been ascribed to either transdifferentiation or cell-cell fusion mechanisms (Frisén, 2002; Kuçi et al., 2009; Pomerantz and Blau, 2004). To date, however, cell fusion events have been considered very rare, and therefore the cell identity of the “newborn” hybrids has never been deeply investigated. Here, we have demonstrated that cell-cell fusion occurs and can be visualized as a very relevant event shortly after transplantation of SPCs into a damaged eye. This is true also after mobilization of c-Kit/Sca1-positive cells from the BM into damaged retinas. In previous studies, the number of hybrids derived from BMC fusion might have been underestimated; indeed, we find here that unless these newly formed hybrids are reprogrammed, they undergo cell death, and therefore they cannot be identified a long time after the transplantation. Moreover, since many hybrids are most likely synkaryons, they cannot be easily detected in sections.

HSPCs fuse with high efficiency with ganglion and amacrine neurons; the resulting newborn hybrids are novel cell entities, and if a Wnt-signaling stimulus is provided, they can initially be transiently reprogrammed, proliferate, and become terminally differentiated neurons. It is remarkable that we found expression of Nanog and Oct4 and, at the same time, expression of Nestin, Noggin, and Otx2 precursor neuronal markers in these hybrids. The expression of Nanog and Oct4 is evidence of reprogramming back to the embryonic stage; however, this state is transient, at least in the case of fusion between HSPCs and retinal neurons. The hybrids very soon commit to neuroectodermal lineage, and indeed, 72 hr after transplantation, Oct4 and Nanog were already downregulated to a certain extent. Finally, in 4 weeks, the hybrids differentiate into ganglion and amacrine neurons, thereby contributing to the regeneration of the retinal tissue. Interestingly, we also found YFP<sup>+</sup> axons in the optical nerves 1 month after transplantation, which indicates that regenerated ganglion neurons can even project their axons along nerves.

These observations led us to anticipate that Oct4 and Nanog might have a functional role in adult tissues during cell-fusion-

mediated regeneration processes. Expression of these genes in adults is controversial (Shin et al., 2010); however, it might well be that their expression has not been clearly appreciated under some circumstances, probably because it is very transient.

In extremely rare cases, we found expression of GFP, and therefore of the Nanog transgene, without expression of RFP. This result was unexpected and might have arisen from the accidental silencing of the RFP allele in the hybrids or from fusion-mediated reprogramming after mobilization of endogenous BM-derived cells.

ESCs have great plasticity, and here we have identified the dedifferentiation events *in vivo*; i.e., reprogrammed hybrids expressing Nanog after the fusion of retinal neurons with ESCs. ESC/retinal-neuron hybrids are probably fully reprogrammed, as they can differentiate into the three lineages, a feature that HSPC-derived hybrids do not have. ESC-derived hybrids can form reprogrammed clones in culture and form teratoma *in vivo*. In contrast, *in vitro*, we were not able to isolate clones from HSPC/retinal-neuron hybrids, which is consistent with their transient reprogramming and fast commitment to neuroectoderm lineage differentiation. Interestingly, reprogramming of retinal neurons was not observed after fusion of RSPCs, which indicates the lower degree of plasticity of these cells with respect to HSPCs. However, we cannot exclude that pluripotent cells are poorly enriched in our RSPC preparation.

Recently, it has been shown that cardiac fibroblasts can be reprogrammed by overexpression of specific transcription factors *in vivo*, which results in an improvement of cardiac function in mice (Qian et al., 2012; Song et al., 2012). However, to date, neuron dedifferentiation *in vivo* has been considered as relatively difficult. Here, we have demonstrated that neurons can indeed change their developmental stage in a living organism while resident in their own tissue. However, when they fuse with HSPCs, they keep the memory of their neuronal identity, as these newborn hybrids finally differentiate into neurons.

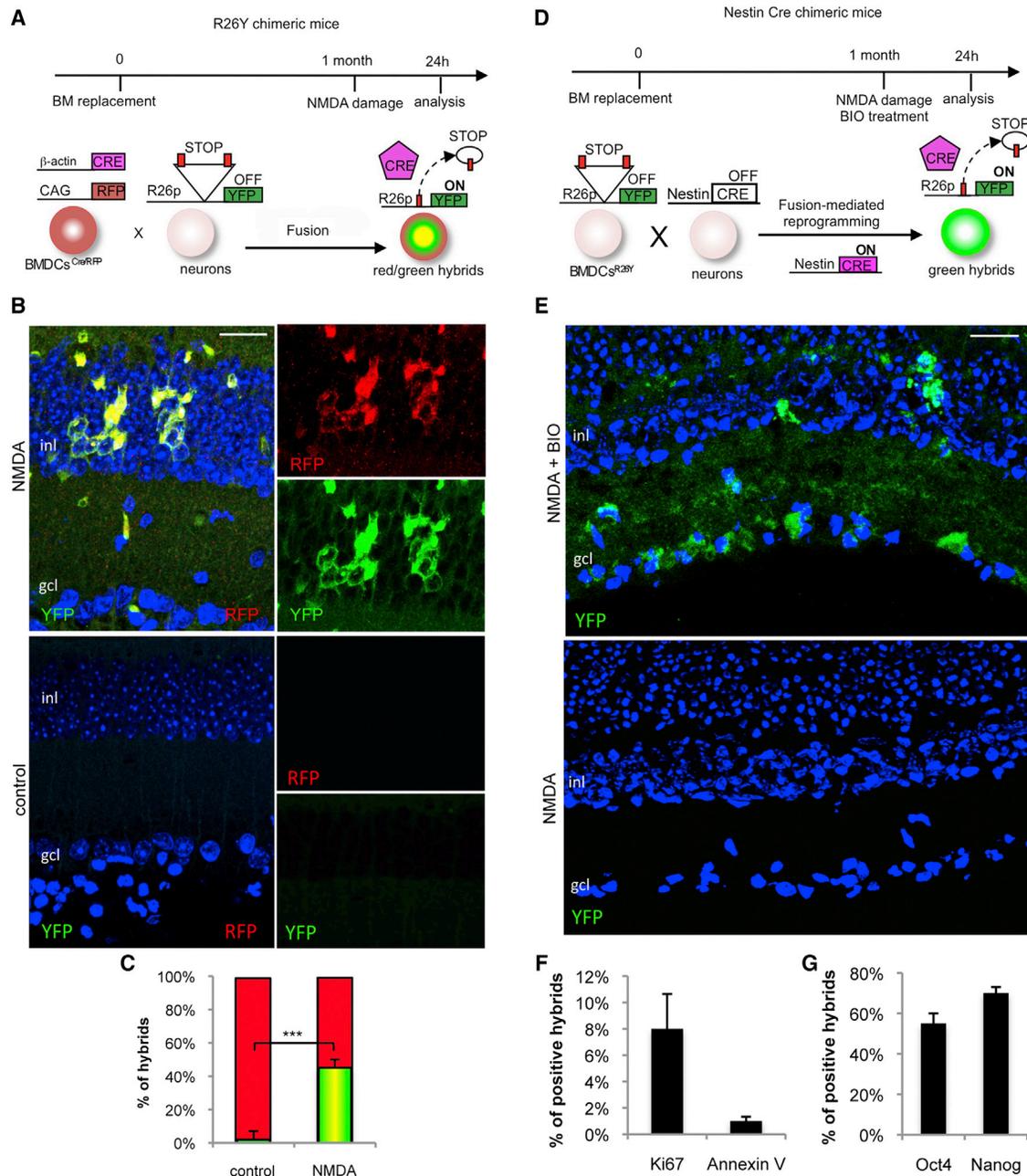
To track *in vivo* reprogramming events is not easy; indeed, these events are transient. Furthermore, ESCs, in principle, do not exist in the embryo or exist for only a few hours. Pluripotent cells, such as the reprogrammed cells, should rapidly undergo a change of fate *in vivo*, which will depend on the different tissue signals and their commitment to progress into a specific differentiation fate. A lineage identity memory that is not erased during the reprogramming process might be beneficial to direct the correct differentiation path *in vivo*. Interestingly, induced pluripotent stem cells have been shown to retain epigenetic memory of their somatic cells of origin (Kim et al., 2010; Polo et al., 2010). Here, in our model, the transition from one cell differentiation stage to another is not direct but passes through the transient re-expression of precursor genes, thereby passing through an intermediate, less-differentiated developmental precursor.

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The light ON/dark bar below the histograms represents the light stimulus that was generated from a computer monitor and was projected onto the retinal surface. ON, ON-OFF, and OFF responses are all recorded by the array. Frequency is in impulses (spikes) per second.

(C) Boxplot latencies to ON peak including (from bottom to top) the smallest observation (sample minimum), lower quartile (25<sup>th</sup> percentile), median, upper quartile (75<sup>th</sup> percentile), and largest observation (sample maximum) of each sampled group.

(D) Normalized responses of ON ganglion cells for control, NMDA-damaged, and NMDA + BIO HSPC retinas as a function of stimulus intensity. Each data point shows the mean  $\pm$ SEM of the percentage of cells responding to each light intensity ranging from black to white (boxes).



**Figure 7. Endogenous BM-Derived Cells Recruited in Damaged Eyes Can Fuse with Retinal Neurons**

(A) Experimental scheme of endogenous BMC-fusion detection. R26Y mice received BM<sup>RFP/Cre</sup> transplantation. After BM reconstitution (1 month), the right eyes received an intravitreal injection of NMDA and the left eyes were not injected. The mice were analyzed 24 hr later. Only in the case of cell fusion of recruited BM cells (red) and neurons were YFP/RFP double-positive hybrids detected.

(B) Double-positive YFP/RFP hybrids detected in NMDA-damaged (NMDA) but not in healthy (control) eyes. Scale bar: 50  $\mu$ m.

(C) Quantification of percentage of YFP<sup>+</sup> hybrids with respect to the total number of detected RFP cells (calculated as for Figure S2B). Data are mean  $\pm$ SEM. \*\*\*p < 0.001.

(D) Experimental scheme of endogenous cell-fusion-mediated reprogramming. Nestin-Cre mice received BM<sup>R26Y</sup> transplantation. After BM reconstitution (1 month), the right eyes received an intravitreal injection of BIO + NMDA, while the contralateral eyes were injected with NMDA alone. Nestin-mediated Cre expression leads to expression of the YFP only in the case of cell-fusion-mediated reprogramming of hybrids between recruited BMCs<sup>R26Y</sup> and neurons.

(E) YFP<sup>+</sup> reprogrammed hybrids (green) after fusion of recruited BMCs and damaged neurons were detected only after BIO injection (NMDA + BIO). In contrast, no YFP<sup>+</sup> hybrids were seen in NMDA-damaged eyes without BIO (NMDA). Scale bar: 50  $\mu$ m.

(F and G) Quantification of percentages of mitotic (Ki67<sup>+</sup>) or apoptotic (Annexin V<sup>+</sup>) hybrids (F) and Oct4-positive and Nanog-positive hybrids (G), evaluated with the respect to the total number of YFP<sup>+</sup> cells. Data are mean  $\pm$ SEM (n = 30).

See also Figure S11.

Wnt signaling controls the regeneration of tissues in response to damage in lower eukaryotes (Lengfeld et al., 2009). Regeneration of the zebrafish tail fin and *Xenopus* limbs requires activation of Wnt/ $\beta$ -catenin signaling (Lin and Slack, 2008); likewise for tissue regeneration in planarians (De Robertis, 2010). Interestingly, in fish and postnatal chicken retina, downregulation of Müller-cell-specific markers, such as glutamine synthetase, and activation of progenitor markers, such as Pax6 and Chx10, have been associated with a regenerative potential of these cells (Fischer, 2005). The regenerative response in zebrafish retina was shown to be due to Müller glia dedifferentiation, which re-expresses pluripotency factors (Ramachandran et al., 2010). However, exogenous activation of Wnt signaling is necessary to induce Müller cell dedifferentiation in mouse retina (Osakada et al., 2007). The Wnt signaling regenerative activity that is present in lower eukaryotes might therefore have been lost during evolution.

Although all of these studies highlight the important role of the Wnt/ $\beta$ -catenin signaling pathway in the regeneration process, the biological mechanisms that form the basis of this regeneration were still largely unknown to date. Here, we have shown that at least in mouse retina, regeneration can occur through cell-fusion-mediated reprogramming. On the other hand, there is nonhomogenous regeneration of the transplanted retinas, which indicates that other factors might be used to enhance these processes (e.g., nerve growth factors). Nevertheless, the main features of the electrophysiological responses of regenerated retinas were similar to wild-type control retinas indicating that cell-fusion-mediated reprogramming has a potential to be a therapeutic approach. However, we cannot exclude that in addition to the generation of new neurons, and therefore the bona fide regeneration of the retinal tissue, we might have also induced delayed neuronal degeneration.

It is also interesting to note that the regeneration process cannot be induced by modulation of Wnt signaling pathway alone; in fact, cell-fusion-mediated reprogramming is also essential. Therefore, it can be therapeutically relevant to implement strategies to increase BMC recruitment to the eyes along with the activation of Wnt signaling. We observed that BMCs fuse with Müller cells after damage; therefore, it might be that the Wnt-dependent dedifferentiation of Müller cells that has been reported previously in mouse retina (Osakada et al., 2007) is also contributed by fusion events with recruited BMCs.

The endogenous *in vivo* reprogramming can be a mechanism of damage repair, and minor cellular damages might be repaired through cell-fusion-mediated reprogramming after the recruitment of BMCs. It is also possible that Wnt-mediated reprogramming is a safeguard mechanism after *in vivo* cell fusion. The hybrids that are not reprogrammed undergo apoptosis-mediated cell death. Instead, Wnt-mediated reprogrammed hybrids can survive and proliferate.

There are obviously many questions still open. For example, do the newborn hybrids maintain the double genome content? And if so, do they silence one of the two genome copies? Or alternatively, might the nuclei divide, or is one of the two nuclei excised by the hybrids in a very early stage after fusion? We found double DNA content in the sorted hybrids. Also, we detected the expression of RFP and YFP transgenes derived

from the genomes of the two different fusion partners, which indicates contributions of both genomes in the hybrids. Moreover, we observed proliferation of the reprogrammed hybrids, an indication that they were mononucleate cells or bona fide synkaryons. However, we also found some heterokaryons shortly after fusion. Stable heterokaryons have been seen with Purkinje cell fusion with BM-derived cells, and their numbers were greatly increased upon inflammation (Johansson et al., 2008). In addition, tetraploid cells have also been found recently in wild-type chicken retinas (Morillo et al., 2010). The exact percentages of synkaryons, heterokaryons, and mononucleated neurons remain to be defined.

In conclusion, after Wnt signaling activation, retinal neurons can be reprogrammed after cell fusion with BMCs and the hybrids can contribute to cell regeneration and repair of retinal tissue. Ultimately, this mechanism can be exploited for regenerative medicine approaches.

## EXPERIMENTAL PROCEDURES

### Retinal Damage and Drug Treatment

Mice at the age of 3 months were anaesthetized by an intraperitoneal injection of ketamine: medetomidine (80 mg/kg: 1.0 mg/kg). To induce retinal damage, the mice were treated intravitreally with 2  $\mu$ l 20 mmol NMDA (Timmers et al., 2001) (total 40 nmol; Sigma) for 24 hr. To block endogenous activation of Wnt signaling, 2  $\mu$ l DKK1 (5  $\mu$ g/ $\mu$ l) was injected soon after the NMDA treatment. Control eyes received 2  $\mu$ l PBS. For the BrdU incorporation assays, the mice received intraperitoneal BrdU administration at 50 mg/kg body weight every 6 hr for 24 hr.

For further details, please refer to [Extended Experimental Procedures](#) and [Table S7](#).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes [Extended Experimental Procedures](#), 11 figures, and 7 tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.06.015>.

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## REFERENCES

Alvarez-Dolado, M., Pardal, R., Garcia-Verdugo, J.M., Fike, J.R., Lee, H.O., Pfeffer, K., Lois, C., Morrison, S.J., and Alvarez-Buylla, A. (2003). Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 425, 968–973.

- Blau, H.M., Chiu, C.P., and Webster, C. (1983). Cytoplasmic activation of human nuclear genes in stable heterocaryons. *Cell* 32, 1171–1180.
- Blau, H.M., Pavlath, G.K., Hardeman, E.C., Chiu, C.P., Silberstein, L., Webster, S.G., Miller, S.C., and Webster, C. (1985). Plasticity of the differentiated state. *Science* 230, 758–766.
- Cowan, C.A., Atienza, J., Melton, D.A., and Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science* 309, 1369–1373.
- De Robertis, E.M. (2010). Wnt signaling in axial patterning and regeneration: lessons from planaria. *Sci. Signal.* 3, pe21.
- Fischer, A.J. (2005). Neural regeneration in the chick retina. *Prog. Retin. Eye Res.* 24, 161–182.
- Frisén, J. (2002). Stem cell plasticity? *Neuron* 35, 415–418.
- Johansson, C.B., Youssef, S., Koleckar, K., Holbrook, C., Doyonnas, R., Corbel, S.Y., Steinman, L., Rossi, F.M., and Blau, H.M. (2008). Extensive fusion of haematopoietic cells with Purkinje neurons in response to chronic inflammation. *Nat. Cell Biol.* 10, 575–583.
- Kim, K., Doi, A., Wen, B., Ng, K., Zhao, R., Cahan, P., Kim, J., Aryee, M.J., Ji, H., Ehrlich, L.I., et al. (2010). Epigenetic memory in induced pluripotent stem cells. *Nature* 467, 285–290.
- Kuçi, S., Kuçi, Z., Latifi-Pupovci, H., Niethammer, D., Handgretinger, R., Schumm, M., Bruchelt, G., Bader, P., and Klingebiel, T. (2009). Adult stem cells as an alternative source of multipotential (pluripotential) cells in regenerative medicine. *Curr. Stem Cell Res. Ther.* 4, 107–117.
- Lengfeld, T., Watanabe, H., Simakov, O., Lindgens, D., Gee, L., Law, L., Schmidt, H.A., Ozbek, S., Bode, H., and Holstein, T.W. (2009). Multiple Wnts are involved in Hydra organizer formation and regeneration. *Dev. Biol.* 330, 186–199.
- Li, V.S., Ng, S.S., Boersema, P.J., Low, T.Y., Karthaus, W.R., Gerlach, J.P., Mohammed, S., Heck, A.J., Maurice, M.M., Mahmoudi, T., and Clevers, H. (2012). Wnt signaling through inhibition of  $\beta$ -catenin degradation in an intact Axin1 complex. *Cell* 149, 1245–1256.
- Lin, G., and Slack, J.M. (2008). Requirement for Wnt and FGF signaling in *Xenopus* tadpole tail regeneration. *Dev. Biol.* 316, 323–335.
- Lluis, F., and Cosma, M.P. (2009). Somatic cell reprogramming control: signaling pathway modulation versus transcription factor activities. *Cell Cycle* 8, 1138–1144.
- Lluis, F., and Cosma, M.P. (2010). Cell-fusion-mediated somatic-cell reprogramming: a mechanism for tissue regeneration. *J. Cell. Physiol.* 223, 6–13.
- Lluis, F., Pedone, E., Pepe, S., and Cosma, M.P. (2008). Periodic activation of Wnt/ $\beta$ -catenin signaling enhances somatic cell reprogramming mediated by cell fusion. *Cell Stem Cell* 3, 493–507.
- Lluis, F., Pedone, E., Pepe, S., and Cosma, M.P. (2010). The Wnt/ $\beta$ -catenin signaling pathway tips the balance between apoptosis and reprogramming of cell fusion hybrids. *Stem Cells* 28, 1940–1949.
- Lluis, F., Ombrato, L., Pedone, E., Pepe, S., Merrill, B.J., and Cosma, M.P. (2011). T-cell factor 3 (Tcf3) deletion increases somatic cell reprogramming by inducing epigenome modifications. *Proc. Natl. Acad. Sci. USA* 108, 11912–11917.
- Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* 20, 781–810.
- MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/ $\beta$ -catenin signaling: components, mechanisms, and diseases. *Dev. Cell* 17, 9–26.
- Morillo, S.M., Escoll, P., de la Hera, A., and Frade, J.M. (2010). Somatic tetraploidy in specific chick retinal ganglion cells induced by nerve growth factor. *Proc. Natl. Acad. Sci. USA* 107, 109–114.
- Niida, A., Hiroko, T., Kasai, M., Furukawa, Y., Nakamura, Y., Suzuki, Y., Sugano, S., and Akiyama, T. (2004). DKK1, a negative regulator of Wnt signaling, is a target of the  $\beta$ -catenin/TCF pathway. *Oncogene* 23, 8520–8526.
- Nirenberg, S., and Meister, M. (1997). The light response of retinal ganglion cells is truncated by a displaced amacrine circuit. *Neuron* 18, 637–650.
- Nygren, J.M., Liuba, K., Breitbach, M., Stott, S., Thorén, L., Roell, W., Geisen, C., Sasse, P., Kirik, D., Björklund, A., et al. (2008). Myeloid and lymphoid contribution to non-haematopoietic lineages through irradiation-induced heterotypic cell fusion. *Nat. Cell Biol.* 10, 584–592.
- Ogle, B.M., Cascalho, M., and Platt, J.L. (2005). Biological implications of cell fusion. *Nat. Rev. Mol. Cell Biol.* 6, 567–575.
- Osakada, F., Ooto, S., Akagi, T., Mandai, M., Akaike, A., and Takahashi, M. (2007). Wnt signaling promotes regeneration in the retina of adult mammals. *J. Neurosci.* 27, 4210–4219.
- Otani, A., Dorrell, M.I., Kinder, K., Moreno, S.K., Nusinowitz, S., Banin, E., Heckenlively, J., and Friedlander, M. (2004). Rescue of retinal degeneration by intravitreally injected adult bone marrow-derived lineage-negative hematopoietic stem cells. *J. Clin. Invest.* 114, 765–774.
- Polo, J.M., Liu, S., Figueroa, M.E., Kulalert, W., Eminli, S., Tan, K.Y., Apostolou, E., Stadtfeld, M., Li, Y., Shioda, T., et al. (2010). Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat. Biotechnol.* 28, 848–855.
- Pomerantz, J., and Blau, H.M. (2004). Nuclear reprogramming: a key to stem cell function in regenerative medicine. *Nat. Cell Biol.* 6, 810–816.
- Qian, L., Huang, Y., Spencer, C.I., Foley, A., Vedantham, V., Liu, L., Conway, S.J., Fu, J.D., and Srivastava, D. (2012). In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature* 485, 593–598.
- Ramachandran, R., Fausett, B.V., and Goldman, D. (2010). Ascl1a regulates Müller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. *Nat. Cell Biol.* 12, 1101–1107.
- Sasahara, M., Otani, A., Oishi, A., Kojima, H., Yodoi, Y., Kameda, T., Nakamura, H., and Yoshimura, N. (2008). Activation of bone marrow-derived microglia promotes photoreceptor survival in inherited retinal degeneration. *Am. J. Pathol.* 172, 1693–1703.
- Shin, D.M., Liu, R., Klich, I., Ratajczak, J., Kucia, M., and Ratajczak, M.Z. (2010). Molecular characterization of isolated from murine adult tissues very small embryonic/epiblast like stem cells (VSEs). *Mol. Cells* 29, 533–538.
- Song, K., Nam, Y.J., Luo, X., Qi, X., Tan, W., Huang, G.N., Acharya, A., Smith, C.L., Tallquist, M.D., Neilson, E.G., et al. (2012). Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature* 485, 599–604.
- Streilein, J.W., Ma, N., Wenkel, H., Ng, T.F., and Zamiri, P. (2002). Immunobiology and privilege of neuronal retina and pigment epithelium transplants. *Vision Res.* 42, 487–495.
- Sullivan, S., and Eggan, K. (2006). The potential of cell fusion for human therapy. *Stem Cell Rev.* 2, 341–349.
- Tada, M., Tada, T., Lefebvre, L., Barton, S.C., and Surani, M.A. (1997). Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *EMBO J.* 16, 6510–6520.
- Tada, M., Takahama, Y., Abe, K., Nakatsuji, N., and Tada, T. (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Curr. Biol.* 11, 1553–1558.
- Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E., and Scott, E.W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* 416, 542–545.
- Timmers, A.M., Zhang, H., Squitieri, A., and Gonzalez-Pola, C. (2001). Subretinal injections in rodent eyes: effects on electrophysiology and histology of rat retina. *Mol. Vis.* 7, 131–137.
- Vassilopoulos, G., and Russell, D.W. (2003). Cell fusion: an alternative to stem cell plasticity and its therapeutic implications. *Curr. Opin. Genet. Dev.* 13, 480–485.
- Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S., and Grompe, M. (2003). Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 422, 897–901.
- Weimann, J.M., Charlton, C.A., Brazelton, T.R., Hackman, R.C., and Blau, H.M. (2003). Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains. *Proc. Natl. Acad. Sci. USA* 100, 2088–2093.
- Ying, Q.L., Nichols, J., Evans, E.P., and Smith, A.G. (2002). Changing potency by spontaneous fusion. *Nature* 416, 545–548.