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Genetic modification increases the survival and the neuroregenerative properties of transplanted neural stem cells

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Abstract

Cell therapy raises high hopes for better treatment of brain disorders. However, the majority of transplanted cells often die soon after transplantation and those that survive initially continue to die in the subacute phase, diminishing the impact of transplantations. In this study, we genetically modified transplanted human neural stem cells (hNSCs), from two distant embryonic SCs lines (H9 and RC17) to express one of four pro-survival factors – Hif1a, Akt1, Bcl-2, or Bcl-xl – and studied how these modifications improve short- and long-term survival of transplanted hNSCs. All genetic modifications dramatically increased survival of the transplanted hNSCs. Importantly, three out of four modifications also enhanced the exit of hNSCs from the cell cycle, thus avoiding aberrant growth of the transplants. Bcl-xl expression provided the strongest protection of transplanted cells, reducing both immediate and delayed cell death, and stimulated hNSC differentiation towards neuronal and oligodendroglial lineages. By designing hNSCs with drug-controlled expression of Bcl-xl, we demonstrated that short-term expression of a pro-survival factor can ensure the long-term survival of transplanted cells. Importantly, transplantation of Bcl-xl expressing hNSCs into mice suffering from stroke improved behavioral outcome and recovery of motor activity in mice.

Introduction

Cell therapy represents a promising approach to treating brain diseases. Neural stem cells (NSCs) or precursor cells are usually transplanted at the site of injury to restore function in damaged brain tissue. Although such transplantations have provided some positive effects in several animal models of brain diseases, they are also accompanied by a high death rate of transplanted cells (reviewed in (1, 2)). The debris from dead cells not only stimulates inflammation around transplants, but also and more importantly, eventually negates the positive effect of transplantations. Two distinct phases of cell death are characteristic for predifferentiated transplanted cells: immediate cell death within the first week after transplantation and continuous cell death over the following time period. Thus, the bulk of transplanted cells often die during the first phase, and the majority of initially surviving cells die progressively during the following weeks/months (3–6). In other cases, when undifferentiated or partially differentiated stem cells of embryonic or pluripotent stem cell (ESC or PSC) origin are transplanted, it is difficult to evaluate cell survival due to the high proliferation rate of transplanted cells. However, uncontrolled proliferation of transplanted cells is undesirable in cell therapy since it might result in the formation of brain tumors (7, 8). Thus, it is important to increase survival of transplanted cells without affecting their proliferation.

Survival of transplanted cells depends both on extrinsic pro-survival signals from surrounding cells and intrinsic survival/death signaling. Attempts have been made to increase the survival of transplanted cells by extrinsic stimulation using growth factors that are secreted by transplanted cells themselves. Although some improvement in survival was observed, growth factor-secreting transplanted cells continued to die, and over time cell death rates became similar to those of nonmodified transplanted cells (9, 10). This can be explained by a loss of growth factor receptors

on transplanted cells. Otherwise, signaling from one growth factor might not be sufficient to stop cell death, and intracellular apoptotic programs eventually override pro-survival signals. Furthermore, the dependence of transplanted cell survival on extrinsic signals from local microenvironment also renders these cells vulnerable to cell degeneration induced by pro-death factors released from the injured area, and transplanted cells could also acquire the phenotype of surrounding diseased cells (11).

Stimulating cell-autonomous pro-survival programs that do not rely on extracellular signaling represents another means of enhancing transplanted cell survival. Indeed, activating intracellular signaling molecules involved in cell survival should efficiently inhibit cell death since such molecules act downstream of extracellular signals in pro-survival cascades.

In this study, we selected four pro-survival molecules that were previously shown to protect neurons during developmental cell death (12), Hif1a, Akt1, Bcl2 and Bcl-xl, and expressed each of their genes in predifferentiated human NSCs (H9 and RC17 hNSCs) that were transplanted in mouse brains. For all four types of genetically modified hNSCs, survival was significantly enhanced in comparison to control cells, reaching up to ~141-fold higher levels for RC17-derived hNSCs. Importantly, for most genetic modifications, numbers of cells that did not exit the cell cycle already 1 month post-transplantation were only negligible, and thus there was no uncontrolled growth of the transplants. To identify the period of transgene expression that is required for pro-survival effects, we transduced hNSCs with a lentivirus in which transgene expression could be activated by doxycycline (Dox). We showed that expression of a pro-survival factor for only 1 week after transplantation is sufficient for long-term cell survival in the transplant. Finally, we confirmed that these transplants can be applied to treat disease in a mouse model of stroke, demonstrating that genetically modified hNSCs improve neurological deficit in brain disease.

Results

Genetic modification of H9 hNSCs enhances their survival

To analyze survival of transplanted cells, we employed human H9-derived NSCs (later H9 hNSCs) that were infected with either control lentivirus or a lentivirus expressing a pro-survival gene (Figure 1A). This cell type for transplantation was chosen because (1) such neural stem/precursor cells are used quite frequently in brain transplantation studies and (2) these cells have the potential to differentiate into neuronal or glial cells, two main cell types that are damaged in brain injuries. We confirmed the differentiation potential of these cells by differentiating them into neurons or glial cells *in vitro* (Figure 1B and data not shown, respectively). To stimulate cell-autonomous survival of transplanted cells, we selected 4 genes that are involved in regulating neuronal survival during prenatal and/or postnatal neurogenesis (13–18), namely, *Akt1*, *Hif1a*, *Bcl2,* and *Bcl2l1* (=Bcl-xl protein). We employed myrAkt1 (19), the constitutively active version of Akt1, to strongly stimulate Akt1 signaling. We then cloned the open-reading frames (ORFs) of the pro-survival genes in the lentiviral vector pCDH-CMV-MCS-T2A-EGFP and transduced cultured H9 hNSCs by one of these viruses.

As our aim was to evaluate how genetic modification of hNSCs affects transplanted cell survival, it was essential that all transplanted cells express the transgene. When transducing millions of hNSCs in adherent cultures, it is difficult to reach an efficiency of cell transduction above 99% for one lentivirus and even more difficult to attain a cotransduction efficiency above 99% when using two or more lentiviruses. This is mainly due to the dilution of viral vector in the culture media during transduction. To overcome this problem, we improved the protocol by transducing cells during the culture splitting, subsequently referred to as “split transduction”, thereby allowing us to transduce up to several millions of cells at an efficiency of >99% in a short time (see methods). We

implemented “split transduction” to infect H9 hNSCs either with empty control pCDH-CMV-MCS-T2A-EGFP lentivirus or a lentivirus expressing one of the pro-survival ORFs, pCDH-CMV-ORF-T2A-EGFP H9 hNSCs. Importantly, 4 days after transduction, we did not observe any noninfected cells, indicating a complete transduction of the transplanted cells (Figure 1C).

After transduction, hNSCs were cultured for 4 more days without growth factors and were transplanted into the striatum of immunodeficient NSG mice. To directly compare survival between control and genetically modified hNSCs, control cells were transplanted into the left and genetically modified cells into the right striatum (Figure 1A). Already 1 week after transplantation, only ~7% of control cells had survived and, by 1 month, survival was decreased to ~5% (Figure 1D, E). Conversely, expression of pro-survival genes dramatically augmented survival of transplanted cells, with an up to 17-fold increase at 3 months post-transplantation (Figure 1D, E).

As mentioned above, there are two distinct phases of cell death in the population of transplanted cells, immediate and continuous cell death. In our experiments, the vast majority of control transplanted cells (>93%) died within the first week after transplantation (Figure 1E). Expression of myrAkt1 was most efficient, resulting in 30% survival of transplanted cells. Three other pro-survival genes, *Hif1a*, *Bcl2,* and *Bcl2l1*, protected transplanted cells to a similar extent, namely, 20% of cells survived (Figure 1E, Supplemental Figure 1). Control cells continued to die and their numbers decreased to 1.8% of initially transplanted cells at 3 months post-transplantation. Importantly, *Akt1*, *Bcl2,* and *Bcl2l1* not only significantly protected transplanted cells from immediate death, but also abrogated continuous cell death (Figure 1E, Supplemental Figure 1). After 1 week, the population of surviving cells expressing *Akt1*, *Bcl2,* or *Bcl2l1* was stable, although *Akt1*- and *Bcl2*-expressing transplants exhibited a small decrease in cell survival between 1 week and 1 month after transplantation. Interestingly, at 1 month post-transplantation, the number of surviving *Hif1a*-expressing cells dropped from 20% to 6.3% (Figure 1E), whereas from 1 month

post-transplantation onwards, cell death in these transplants was inhibited. Thus, it is likely that *Hif1a* expression only delayed immediate cell death, but abolished continuous cell death. The pro-survival effect by genetic modification could also be reproduced in immunosuppressed wild-type mice that received either EGFP-only or Akt1-expressing transplants of H9 hNSCs (Supplemental Figure 2).

To analyze whether a combination of pro-survival genes could enhance survival of transplanted cells better than a single pro-survival gene, we induced expression of the three most efficient pro-survival factors, namely, *Akt1*, *Bcl2,* and *Bcl2l1*, in H9 hNSCs and transplanted genetically modified cells in the NSG mice. Strikingly, three-factor (=3f in Figure 1E, Supplemental Figure 1) stimulation had effects on cell survival similar to those of myrAkt1 alone, indicating that all three factors most likely act via the same signaling pathway.

To confirm that expression of pro-survival genes decreases cell death, we analyzed the apoptotic marker activated caspase-3 in transplanted cells. At 5 days post-transplantation, control transplants contained many activated caspase-3-positive cells and some of them still expressed EGFP (Figure 2A-C). Notably, expression of the apoptotic marker in genetically modified cells was hardly detectable (Figure 2A-C).

Genetically modified transplanted H9 hNSCs exit the cell cycle and differentiate

Uncontrolled proliferation of transplanted cells can lead to tumor formation; therefore, we investigated how soon transplanted cells exit the cell cycle. To this end, we colabeled transplanted H9 hNSCs with Ki67, a marker that is expressed only in cells that re-entered the cell cycle. Specificity of Ki67 labeling was confirmed by strong labeling of cycling neural precursor cells in the subventricular zone (SVZ) (Figure 3A, B), a site of postnatal neurogenesis in mice (20, 21). By 1 month post-transplantation, only very few Ki67-positive cells were found in the transplants

(Figure 3A). Furthermore, these cells often did not coexpress EGFP or human nucleus antigen (HuNuc) (Figure 3C), suggesting that a portion of Ki67-positive cells represent endogenous glial cells that were activated upon transplantation. Indeed, among the genetically modified cells that expressed *Hif1a*, *Bcl2,* or *Bcl-xl*, Ki67 was only expressed in 1 out of 1000 cells by 3 months post-transplantation (Figure 3D, E). Importantly, those cells that expressed *Akt1* included a significantly higher number of Ki67-positive cells (Figure 3E), and three-factor-expressing cells showed a similar number of Ki67-positive cells, indicating that these cells resemble cells that express *Akt1* only (Figure 3E). Thus, it is possible that the number of surviving myrAkt1-positive cells is somewhat overestimated since some transplanted cells might continue to proliferate. Additionally, newly generated *Akt1*-expressing cells might replace some dying cells in the transplant, since in spite of relatively high number of Ki67-positive cells, *Akt1*-expressing grafts do not growth with time. Moreover, we never observed teratomas arising from *Akt1*-expressing transplants within the time frames of our analysis.

To analyze whether expression of pro-survival genes change the differentiation profile of transplanted cells, we colabeled transplants with markers for immature and mature neural cells. All genetically modified transplants except those expressing *Hif1a* contained higher numbers of nestin-positive cells, a marker of neural precursor cells, than controls at 3 months post-transplantation (Figure 4A). However, as shown above, in the case of *Bcl2* and *Bcl2l1* expression, it did not affect the number of Ki67-positive cells (see Figure 3E). Thus, although ~10% of *Bcl2* and *Bcl2l1*-expressing cells still continue to express nestin at 3 months post-transplantation, these cells do not express Ki67 and had exited the cell cycle. Strikingly, only Bcl-xl enhanced neuronal differentiation as indicated by colabeling with NeuN, a neuronal differentiation marker (Figure 4B). Interestingly, in neuronal differentiation, threefactor-expressing transplants resembled *Bcl2l1*-expressing cells rather than *Akt1* cells (Figure 4B). Both *Bcl2l1* and *Akt1* expression also enhanced

oligodendrocyte differentiation (Figure 4C), and none of the transplanted cells differentiated into GFAP-positive astrocytes, at least by 3 months after transplantation (Figure 4D).

Short-term induction of expression of a pro-survival factor enhances long-term survival of transplanted H9 hNSCs

In some instances, it is not desirable in the long term to stimulate expression of a certain factor when sustained signaling by the factor triggers unwanted effects, e.g., enhanced proliferation or retarded differentiation. In order to control expression of transgenes in genetically modified H9 hNSCs, we designed an inducible lentiviral vector (Figure 5A). It had two parts, one expressing rtTA3 under the constitutively active promoter EF1 and the other coding for a gene of interest linked to EGFP via a T2A sequence expressed under the TRE-responsive or "Tet-On" promoter. Thus, upon addition of doxycycline (Dox), rtTA3 activates the Tet-On promoter and drives the expression of the gene of interest together with EGFP. We tested this lentivirus *in vitro* by transducing H9 hNSCs and activating EGFP expression with Dox (Figure 5B). Without Dox, EGFP was not expressed in the transduced cells. However, 1 day after adding Dox to the cell medium, strong EGFP expression was observed in every cultured cell. Two days after withdrawing Dox, EGFP expression was weak and disappeared by 8 days post-withdrawal (Figure 5B).

We further confirmed applicability of the lentivirus *in vivo* by directly injecting it into the subventricular zone (SVZ) of adult mouse brains. It is known that neuroblasts produced in the SVZ migrate towards the olfactory bulb and mature there into interneurons (13, 22). To label these cells, we put Dox in the drinking water of the mice 20 days post-injection and analyzed EGFP expression (Figure 5C). The olfactory bulb did not contain any EGFP-positive cells before giving Dox, whereas by 3 days on Dox, numerous EGFP-positive cells could be detected (Figure 5C). Thus, both *in vitro* and *in vivo* experiments provided evidence that, in the infected cells, expression of the

gene of interest could be regulated using our inducible vector system.

To analyze whether transient expression of a pro-survival gene enhances transplanted cell survival similarly to constitutive expression, we transduced H9 hNSCs with a lentivirus expressing rtTA3 and *Bcl2l1* together with EGFP. We transplanted genetically modified H9 hNSCs into the striatum of NSG mice that were pretreated for 3 days with Dox. At 7 days post-transplantation, all H9 hNSCs expressed EGFP and Bcl-xl (Figure 5D). Importantly, survival of transplanted H9 hNSCs that exhibited Dox-regulated *Bcl2l1* expression (Figure 5E) was comparable to those H9 hNSCs with permanent *Bcl2l1* expression (Figure 1E). There was a slight decrease in survival of *Bcl2l1*-expressing H9 hNSCs at 1 month post-transplantation in mice that continuously received Dox (Figure 5E), similar to permanent Bcl-xl overexpression. However, and most importantly, Dox withdrawal at 7 days post-transplantation did not affect survival of transplanted cells analyzed either 7 days or 28 days after withdrawal (Figure 5E). Thus, upregulation of the pro-survival pathway for just 7 days after transplantation is enough to trigger long-term survival of transplanted cells.

Genetic modulation of survival can be applied to hNSCs from different sources

To investigate if modulation of pro-survival factors is applicable to hNSCs in general, we differentiated hNSCs from a clinically relevant hESC line (RC17), which has been derived under Good Manufacturing Practice (GMP). The RC17-derived hNSCs were after differentiation maintained under the same culturing conditions as had been used for the H9 hNSCs, and we confirmed that the RC17 hNSCs expressed markers of forebrain NSCs (OTX2, PAX6, SOX1 and N-cadherin, Figure 6A, B). From the study with H9 NSCs, we found Bcl-xl and Bcl-2 to be the most promising factors for hNSC survival modulation in terms of translational potential, since they provided strong and persistent pro-survival effect accompanied by fast cell cycle exit. Therefore,

we genetically modified RC17 hNSCs with lentiviruses to overexpress either *Bcl2l1* or *Bcl2* and compared them to RC17 hNSCs expressing EGFP only. Similar to H9 hNSCs, all infected RC17 hNSCs expressed EGFP, indicating a complete transduction (Figure 6C). Transduced RC17 hNSCs were cultured for 4 days without growth factors followed by transplantation into the striatum of immunodeficient NSG mice. Control cells were transplanted into the left and genetically-modified cells into the right striatum (Figure 6A). Strikingly, the pro-survival effect of both *Bcl2l1* and *Bcl2* was even stronger for RC17 hNSCs than for H9 hNSCs. Thus, intrinsic survival potential of RC17 hNSCs was much lower than of their H9 hNSCs counterparts – only 0.62% and 0.14% of control RC17 hNSCs survived 1 week and 1 month post-transplantation, respectively (Figure 6D, E), opposite to ~7% and ~5% for H9 hNSCs (Figure 1D, E). Furthermore, already at 1 month post-transplantation, we noticed complete cell death of 2 control RC17 hNSC transplants, which was observed for H9 hNSCs not before 3 months post-transplantation. Nevertheless, modulation of *Bcl2l1* or *Bcl2* expression, increased survival of RC17 hNSCs to the levels that were similar to H9 hNSCs (Figure 6D, E). Thus, by calculating fold increase in survival, we show an 147- and 131-fold enhancement in 1 month survival of transplanted RC17 hNSCs by modulating *Bcl2* and *Bcl2l1* expression, respectively.

To analyze whether an active cell cycle could contribute to such dramatic pro-survival effect, we labeled transplanted RC17 hNSCs with anti-Ki67 antibodies. As before, the specificity of Ki67 labeling of actively cycling cells was confirmed by detection of cycling neural precursor cells in the SVZ (Figure 6F). By 1 month post-transplantation, very few Ki67-positive cells were found in the RC17 transplants (Figure 6F, G), indicating that modulation of *Bcl2* or *Bcl2l1* expression has truly pro-survival effect, with little impact of additional proliferation.

In summary, all major effects of pro-survival modulation have been reproduced using RC17-derived hNSCs, including dramatic increase in short- and long-term survival of genetically-

modified transplanted cells and exit from the active cell cycle.

Transplanted hNSCs with genetically enhanced survival improve behavioral abnormalities in a mouse model of stroke

In order to investigate whether genetically enhanced survival of transplanted cells increases the impact of cell therapy in neurological disorders, we utilized a mild mouse model of stroke where the middle cerebral artery is permanently occluded (MCAO) to induce ischemic brain damage. Such distal MCAO model affects cortex focally sparing subcortical regions and mimics the majority of human strokes that occur at the territory of the MCA. In this model, genetically modified hNSCs that are transplanted in the infarct zone should not only positively influence the surrounding brain region but also resist the hostile necrotic environment of the infarct zone. Two days after MCAO, H9 or RC17 hNSCs transduced with the control lentivirus (EGFP-only) or lentivirus-expressing *Bcl2l1* together with EGFP were transplanted into the peri-infarct zone in the brains (Figure 7A). A sham group received sham injection after MCAO. In order to assess the effect of genetically modified cell transplants, the stroke-induced neurological deficit was evaluated using three different sensorimotor function tests 1 day before MCAO and 7 days after cell transplantation. Importantly, both H9 and RC17 hNSCs with enhanced survival led to significant functional improvements in MCAO-affected mice relative to sham and non-modified H9 and RC17 hNSCs (Figure 7B-Q). Furthermore, although there were some specific differences between effects of H9 and RC17 hNSCs with enhanced survival, overall effect was similar for both types of hNSCs. Thus, both H9 and RC17 *Bcl2l1*-expressing cells provided a rather minor effect on functional improvement in the latency-to-move (Figure 7B-E) and sticky-tape test (Figure 7F-I), and the effect was larger for RC17 *Bcl2l1*-expressing cells.

In contrast to previous reports (23), distal MCAO had no effect on sham-injected mice regarding

the sticky-tape and latency-to-move test. Such a lack of effect might be due to depletion of the immune system in the NSG mice as the response of the immune system to an ischemic insult is crucial for development of the injury (24–26). However, a clear stroke-induced deficit in sensorimotor function was detected using a handedness test. While the number of grabbing attempts with the contralateral paw and the Collins score were clearly reduced in sham and EGFP-only groups, mice with *Bcl2l1*-expressing H9 or RC17 cell transplants completely recovered to baseline level in a week after transplantation (Figure 7J-Q). Importantly, the transplantation of control cells had no or very minor effect on recovery in the handedness test (Figure 7J-Q). Thus, *Bcl2l1*-expressing hNSCs of both types successfully promoted the recovery of damaged brain areas responsible for coordinating the forelimbs.

To validate that expression of a pro-survival gene increases survival of transplants in a hostile environment of brain tissue affected by the stroke, we analyzed the transplantation area immediately after the behavioral tests. Since all brains in MCAO models miss large part of the cortex in the area of ischemia due to massive cell death (Figure 8A, damaged area), we quantified the number of transplanted cells surrounding the damaged area. Importantly, while only few control hNSCs were found, genetic modulation of hNSCs dramatically increased the number of hNSCs that survived around the damaged area (Figure 8A-D, only RC17 hNSCs are shown since quantifications could not be done for H9 hNSCs – see Methods). Since we transplanted cells in/nearby the infarct zone, we expect a large part of hNSCs being settled in fragile tissue in the epicenter of damage that could not be recovered during histology. Thus, the actual number of survived hNSCs might be much larger than we observed. Overall, we demonstrated that genetically modified hNSCs are better protected from the hostile environment of the infarct zone than naïve cells, allowing genetically modified cells to have a stronger effect on recovery from injury in neurological disorders.

Discussion

Poor survival of transplanted cells in brain transplantations decreases the efficacy of cell therapy and requires large numbers of transplanted cells to be generated. Here, we demonstrate that survival of transplanted hNSCs can be dramatically improved by stimulating pro-survival pathways, leading to better functional recovery than that of naïve hNSCs in a brain disease model.

Numerous studies show poor survival of transplanted hNSCs in the brain, and often the numbers of cells derived from the transplant do not reflect the pure survival potential of hNSCs, but rather the replenishment and expansion of the cell population due to extensive proliferation of hNSCs, which may result in tumor formation (3–8, 27). In the survival of transplanted cells two phases can be distinguished: so-called short-term survival (or immediate death), which ends by 1 week post-transplantation, and a long-term period (continuous cell death) that persists for months. In our experiments, all four pro-survival factors profoundly inhibited the caspase-3-dependent apoptotic pathway. Expression of *Akt1*, *Bcl2,* and *Bcl2l1* not only significantly protected transplanted cells from immediate death, but also abrogated continuous cell death, while *Hif1a* expression only delayed immediate cell death. Interestingly, the number of *Hif1a*-expressing transplanted cells did not drop between 1 and 3 months post-transplantation, indicating that *Hif1a* expression protected against continuous cell death. Expression of *Hif1a* was also reported to improve survival of mouse NSCs that were transplanted in the brain ventricle (28).

Since nondifferentiated NSCs can become tumorigenic after transplantation (7, 8), we were concerned about possible tumorigenesis in the animals transplanted with hNSCs expressing pro-survival factors. In order to decrease the proliferating potential of hNSCs, we initiated cell differentiation already in the culture by withdrawing growth factors from the media. Importantly,

for 3 out of 4 pro-survival factors, <0.5% of Ki67-positive cells were observed at 1 month post-transplantation and ~0.1% at 3 months post-transplantation.

Availability of pro-survival factors varies within the brain and the response to different pro-survival factors depends on the stages of neuronal maturation (for review, see (12)). Thus, we chose four pro-survival factors, Hif1a, Akt1, Bcl2, and Bcl-xl, which have the potential to stimulate survival of neural cells already very early during cell differentiation. Akt1 signaling regulates many cellular processes, including pro-survival mechanisms via regulation of a large number of substrates such as p53, mammalian target of rapamycin (mTOR), and caspase-3/caspase-9, thus contributing to anti-apoptotic signaling in various cell types (29, 30). Extensive data show that Akt1 regulates pro-survival signaling already in precursors, not only in immature neurons (31–33). For instance, mTOR, which is regulated by Akt1 (30), controls cell cycle length and exit in several types of cells, including neuronal progenitor cells, and knockout of *Mtor* results in a dramatic decrease in numbers of precursor cells (34). However, it may be difficult to distinguish between the regulation of cell death and other intracellular pathways, and increased expression of Akt1 can result in too broad of a spectrum of effects, depending on current cell fate and the environment surrounding cells. Furthermore, although in our study Akt1 overexpression resulted in dramatic increase in transplanted cell number, Akt1-expressing cells kept being in active cell cycle for much longer than other genetically-modified cells, thereby diminishing translational potential of Akt1-expressing cells due to potential teratogenic effects.

The Bcl-2 protein family acts as key regulators in the intrinsic or mitochondrial apoptosis pathway. The family is comprised of two types of members: those that prevent apoptosis (Bcl-2 and Bcl-xl) (35) and those that promote apoptosis (Bax, Bid, and Hrk) (35). Systemic delivery of Bcl-xl fusion protein inhibited caspase-3 and -9 activities and also prevented translocation of apoptosis-inducing factor AIF into the nucleus following hypoxic-ischemic brain injury (36). In our experiments, both

Bcl2- and *Bcl2l1*- expressing transplanted cells exhibited a high survival rate and the transplanted cells exited the cell cycle soon after the transplantation. *Bcl2l1,* but not *Bcl2* expression also enhanced neuronal differentiation of the transplanted cells. Interestingly, a recent study showed that *Bcl2* expression also protected cortical neuron progenitors from cell death *in vitro* (37), suggesting that Bcl-2 family proteins are good candidates for neuroprotection at various stages of neuronal differentiation. Importantly, we showed in the current study that *Bcl2l1* expression only for 1 week after transplantation protected transplanted neuronal progenitors for more than 1 month *in vivo*. The ability to regulate gene expression in the transplanted cells is one of the most critical issues for successfully implementing gene therapies. Our results demonstrate that short-term expression of a transgene in the transplanted cells could have long-lasting effects. A similar system can be used to quickly turn transgene expression "off" and "on" in transplanted cells when necessary, thus enhancing the safety of the cell therapy.

Cerebral ischemia is characterized by oxidative stress, an inflammatory response, and apoptosis in the infarct core and penumbra zone (62). Thus, it is an excellent model to test the potential of genetically modified hNSCs that are transplanted in the necrotic environment and experience strong prodeath signaling from the environment. The immediate, positive effect of transplanted NSCs after stroke is mediated by the secretion of trophic pro-survival factors affecting surrounding cells (38). However, a severe limitation of treating ischemic brain injuries with exogenous hNSCs is that only a small number of cells could survive after transplantation in the damaged tissue. Here, we injected hNSCs at 48 h after distal, permanent MCAO in/nearby the peri-infarct zone. Very few control cells survived in the hostile environment, whereas survival of *Bcl2l1*-expressing hNSCs was 22-fold higher. Moreover, *Bcl2l1*-expressing, but not control hNSCs improved the deficit in sensorimotor function, reflecting an improved outcome in these mice. Thus, results suggest that in order to reach functional benefits from transplantation in the stroke model

much fewer genetically modified hNSCs could be transplanted (50,000 genetically modified cells instead of 1,000,000 naïve cells by approximation of ~20-fold increase in survival *Bcl2l1*-expressing cells). Importantly, all major effects of pro-survival modulation were reproduced in two different stem cells lines, H9- and RC17-hNSCs, which increase the robustness of the results and therapeutic relevance of our approach.

Stem cell therapy offers the possibility of a renewable source for replacing cells and tissues to treat a long list of diseases, conditions, and disabilities, including Parkinson's and Alzheimer's diseases, spinal cord injury, stroke, cerebral palsy, amyotrophic lateral sclerosis, loss of vision, and other neurodegenerative diseases. However, up to 98% of transplanted hNSCs die within 1-2 months after transplantation (27, 39). Therefore, tens of millions of cells are currently required for human cell transplantation therapies due to the low survival, which reduces the feasibility of cultivating such enormous numbers of cells. Moreover, massive death of the transplanted cells will induce an additional inflammatory reaction in the injured brain areas, thus providing negative instead of positive effects for the diseased brain. Enhancing survival of transplanted cells in cell therapy for brain disorders would not only improve the outcome of cell therapy and avoid additional injury from the transplantations themselves, but also dramatically decrease the number of cells needed to be generated for cell therapy.

Methods

Animals

For all of the experiments, we used immunodeficient NSG (NOD scid gamma) mice (40) and wild-type C57Bl/6 mice. All experimental procedures involving mice were performed according to the regulations of Copenhagen University and Luebeck University (Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume, Kiel, Germany). Mice were housed under standard

conditions (12h/12h light/dark cycle, access to dry food and water ad libitum). Both males and females were used throughout the study.

Molecular cloning

The pCDH-CMV-MCS-T2A-EGFP vector was generated by modifying pCDH-EF1-MCS-T2A-copGFP (System Bioscience, USA), where the EGFP and CMV cassettes were subcloned from pEGFP-C1 (Clontech-Takara Bio) and the T2A sequence was subcloned from pCDH-CMV-T2A (41). The shuttle expression plasmids containing ORFs of mouse *Akt1*, *Hif1a*, *Bcl2,* and *Bcl2l1* (=Bcl-xl protein) genes were ordered from Biocat and subcloned into the MCS of the pCDH-CMV-MCS-T2A-EGFP vector.

Recombinant lentivirus production and titration

Lentiviruses were produced as previously described (21). Human embryonic kidney 293 cells (HEK 293 cells, Gibco) were maintained in growth medium (DMEM, 2mM Glutamine, 10% FCS, 1 % Pen/Strep – all from Gibco) and incubated at 37°C, 5% CO_2. The day before transfection, HEK cells were plated on 12×10-cm plates (4×10^6 cells/plate), so that the cells were approximately 80% confluent at the time of transfection. Growth medium was changed to transfection medium (DMEM, 2 mM Glutamine) 1 h before transfection. The cells were transfected by using the calcium–phosphate method with four plasmids: recombinant lentiviral expression vector (10 μg/plate) and three helper plasmids (pRev, pRRE, and MD2.G, 5 μg of each helper per plate). Plasmid DNA was mixed with $CaCl_2$ (final concentration 0.14 M) and 2×HBS (final: 280 mM NaCl, 1.5 mM $NaH_2PO4(\times2H_20)$, pH 6.96) with a final volume of 12 ml of the mixture. The mixture was added directly to transfection medium (1 ml per plate containing cells) and incubated 4-5 h at 37ºC and low-level CO_2 (3%). Medium was changed and cells were incubated in growth

medium at 37ºC, 5% CO_2, for 60-72 h after transfection to generate and release viral particles in the media. Following this period, the supernatant from plates was collected and cleared of debris by low-speed centrifugation (200 × *g*, 5 min), filtered through 0.25 μM filters, spun at 120 000 × *g* for 1.5 h (Beckman Coulter Optima ultracentrifuge, rotor SW28) and the pellet was resuspended in PBS.

The titers of viruses were determined by infecting HEK 293 cells seeded in 96-well plates at 4×10^4 cells/well the day before infection with serial dilutions of the virus stocks in transfection medium. After overnight incubation, the cell culture medium was changed, and 48 h later, the number of infected cells and the actual viral titer were scored by counting EGFP-positive cells across dilutions of viral stock.

hNSC culture

Human H9 (WiCell, hPSCreg WAe009-A)-derived neuronal stem cells hNSC (Gibco) were cultured as a monolayer on plasticware (Nunc) coated with CELLstart (Gibco) in growth media containing KnockOUT™ DMEM/F-12 supplemented with 2% StemPro® Neural Supplement, 20 ng/ml FGFb and 20 ng/ml EGF, 2 mM GlutaMAX™, and 1% Antibiotic-Antimycotic (cells and culture reagents were obtained from Life Technologies); the medium was changed every 2 days. For plating of hNSC, 10 μM ROCK inhibitor (Selleckchem) was added to the medium and 24 h after plating, the medium was changed to growth medium without ROCK inhibitor. When growth was approximately 90% confluent, cells were split with StemPro Accutase (Life Technologies).

An alternative source of hNSCs was generated from the human embryonic stem cell line RC17 (Roslin Cells, hPSCreg RCe021-A). RC17 hESCs were maintained on plates coated with growth factor-reduced Matrigel (Fisher Scientific) in StemMACS iPS-Brew XF (Miltenyi Biotec). Cells were passaged with EDTA (0.5 mM) and supplemented with ROCK inhibitor (10 μM Y-27632)

on days of passaging. For differentiation into NSCs, RC17 hESCs were seeded at 10,000 cells/cm^2 onto Matrigel-coated plates and cultured for 9 days in N2 medium (1:1 mix of DMEM/F12:Neurobasal with 1% N2 supplement and 200 mM L-Glutamine) supplemented with 10 μM SB431542, 100 ng/ml rhNoggin (i.e. dual SMAD inhibition) and 100 ng/ml FGF8b for dorsal forebrain specification. On day 9, the cells were passaged and cultured from day 9-15 in N2 medium with 1% B27 supplement, 20 ng/ml EGF and 20 ng/ml bFGF. On day 15, cells were passaged again and medium was changed to DMEM/F12 with 2% StemPro neural supplement, 20 ng/ml EGF and 20 ng/ml bFGF. This same medium was used to further expand and maintain the RC17 NSCs. StemPro Accutase was used for passaging the RC17 NSCs and 10 μM ROCK inhibitor was added to the medium on all days of passaging.

Split transduction of hNSCs and preparation for transplantation

In order to achieve a high efficiency of the viral transduction of hNSCs, transduction was performed while splitting hNSCs cultures by using a novel "split transduction" protocol. The cells were detached with StemPro Accutase and centrifuged (5 min, 200 × *g*); the supernatant with cells was resuspended in 1 ml of the growth medium; and the cells were counted and then centrifuged again. Supernatant was removed and 20-30 μl of viral solution in PBS was added to the cell pellet (with MOI=2). Virus and cells were carefully mixed, avoiding bubbles, and an equal amount of growth medium was added to the suspension. Then, cells were incubated at 37ºC for 10 min. As the viral solution might contain debris that can inhibit cell survival, cells were washed in 30 ml of the growth medium before plating, then centrifuged (5 min, 200 × g), resuspended in growth medium, and plated at density of 0.5×10^5 cells/cm^2 in growth medium containing 10 μM ROCK inhibitor. Twenty-four hours after plating, the medium was changed to the growth medium without ROCK inhibitor and growth factors and incubated for 4 days to initiate differentiation of hNSCs. The cells

were collected for transplantation by treatment with StemPro Accutase, followed by centrifugation at 200 × *g* for 5 min and washing the pellet twice by resuspension in D-PBS (without Ca^{2+} and Mg^{2+}) and centrifuged again. Final dilution of cells was in Leibovitz's L-15 medium (Sigma-Aldrich) at a concentration of 50000 cells/µl.

Stereotactic transplantation

Cells were kept on ice for the entire procedure before they were transplanted in the brain. Stereotactic surgery was performed as reported previously (41, 42). NSG mice were pre-anesthetized in an isoflurane chamber and transferred in the stereotactic frame with a gas mask (Angle Two, Leica or Kopf) that had continuous flow of isoflurane. After applying local anesthesia and making a short skin incision, the skull above the transplantation site was labeled and a hole was drilled in the skull with a fine syringe needle. The injections were made with glass capillaries using the following coordinates (from Bregma): A/P +0.9, L/M +1.7, D/V -3.0 (for H9 hNSCs) and A/P +0.5, L/M +1.7, D/V -3.0 (for RC17 hNSCs). Then, 1 µl (50000 cells) was slowly injected into each transplantation site using an oil pressure pump, followed by 3-min incubation of the capillary at the injection site and slow retrieval of the capillary.

C57Bl/6 mice underwent similar procedure for transplantation. To reduce innate immune response that could affect survival of human cells in mouse brain, C57Bl/6 mice were immunosuppressed by daily injections of cyclosporine (15mg/kg/day) and prednisolone (2mg/kg/day) that started 2 days before the transplantations and were performed until sacrificing mice for immunohistochemistry. Note that in spite of immunosuppression, there were a number of transplants (both control and genetically-modified) that were rejected by immune system.

Immunocytochemistry and immunohistochemistry

Immunolabeling was performed as described previously (43, 44). Briefly, for immunocytochemical staining, NSC cultures were fixed by cold 4 % paraformaldehyde (PFA, Merck) for 10 min, washed with PBS, followed by permeabilization with 0.2 % Triton X-100 and blocked with 5 % BSA for 30 min. Primary antibodies were incubated overnight in 0.1 % Triton and 5 % BSA at 4°C, followed by washing with PBS and incubation with secondary antibodies in 0.1 % Triton and 5 % BSA at room temperature.

For immunohistochemistry, mice were deeply anesthetized by injecting ketamine/xylazine intraperitonally and transcardially perfused, and the brains were postfixed in 4% PFA overnight. Brain sections (50 μm) were prepared using a Leica VT1000S vibratome (Leica). Free-floating sections were permeabilized with 0.2 % Triton X-100 and blocked by 5 % BSA and incubated in 0.1 % Triton and 5 % BSA at 4°C with primary antibodies. Sections were washed and secondary antibody incubation was carried out at room temperature for 4 h. Primary antibodies that were used are presented in Table 1.

Table 1. Primary antibodies used for immunocytochemistry and immunohistochemistry

Antibody	Dilution	Company
mouse anti-Beta-tubulin III (Tuj1)	1:500	Sigma-Aldrich
mouse anti-Bcl-x	1:500	Santa Cruz Biotechnology
mouse anti-N-cadherin	1:800	BD Bioscience
rabbit anti-cleaved caspase-3	1:1000	R&D Systems
chicken anti-EGFP	1:1000	Invitrogen
rabbit anti-EGFP	1:1000	Invitrogen
mouse anti-GFAP	1:2000	Sigma-Aldrich

mouse anti-Human Nuclei	1:250	Millipore
rabbit anti-Ki67	1:500	Abcam
goat anti-Luciferase	1:1000	Novus Biologicals
mouse MAP2 antibody	1:500	Pharmingen
rabbit anti-Nestin	1:500	Abcam
chicken anti-NeuN	1:1000	Millipore
mouse anti-NeuN	1:1000	Millipore
goat anti-Olig2	1:500	R&D Biosystems
goat anti-Otx2	1:500	R&D Biosystems
rabbit anti-Pax6	1:200	Boligen
rabbit anti-Sox1	1:100	Cell Signaling

As secondary antibodies we used: Alexa 488-conjugated anti-rabbit, anti-mouse, anti-chicken, and anti-goat; Alexa 546-conjugated anti-mouse and anti-rabbit; Alexa 594-conjugated anti-rabbit; Alexa 647-conjugated anti-mouse and anti-rabbit (1:1000, Invitrogen). DAPI - 4′,6-Diamidine-2′-phenylindole dihydrochloride (Sigma-Aldrich) was used to label cell nuclei.

Double-labeling was quantified confocally, see Supplemental Figure 3 as an example.

Middle cerebral artery occlusion – MCAO

As stroke model we induced permanent focal cerebral ischemia. Here, the distal middle cerebral artery (MCA) was occluded by electrical coagulation in 8-week-old NSG mice (45). First, mice were anesthetized with tribromoethanol (25 µl of 1.5 % tribromoethanol/g bodyweight). Then, the mice were placed under a microscope (Hund) and rectal temperature was maintained at 37° C with a heating pad. A skin incision was made between the ear and the orbit on the left side. After

removing the temporal muscle, a burr hole was drilled and the meninges were removed. The exposed left MCA was occluded by microbipolar electrocoagulation (Model ICC 50; Erbe). Finally, the skin incision was sutured and the mice were placed under a heating lamp until they fully recovered. After 48 h, mice were anesthetized with 1-2 % isoflurane and fixed in a stereotaxic frame (David Kopf instruments; No. 1900) to inject hNSCs into the peri-infarct area (the area of injection matched the area of the infarct zone). The following coordinates were used for injecting the cell suspension (50,000 cells in 1 µl) or vehicle (sham): A/P -2.0, L/M +2,3, D/V -2.5 (for H9 hNSCs) and A/P -0.5, L/M +2,3, D/V -2.0 (for RC17 hNSCs). H9 hNSCs were transplanted closer to the center of the infarct zone and thus the majority of them were expected to reside the damaged cortical area that could not be preserved for histology, thus, precluding reliable calculations of H9 hNSC survival in the MCAO model. RC17 hNSCs were transplanted further away from the center of the infarct zone, and thus their survival could be quantified.

Behavioral tests

In order to evaluate sensorimotor function, three different tests were performed 1 day before MCAO surgery, 7 days after hNSC injection. The latency-to-move and the sticky-tape-removal tests have been described previously (25). For the latency-to-move test, mice were placed at the center of a plain board. The time to cross one body length (7 cm) was measured. In the sticky-tape-removal test, a small circular adhesive tape (HERMA No. 2212, 8 mm) was placed on both forepaws one after another. Then, the time until the mouse recognized and started to remove the pad as well as the total time to remove it were determined. The handedness of the mice was investigated using the Collins apparatus as described by Collins (46, 47). The apparatus is composed of an acrylic glass box which measures 10.2 × 6 × 6 cm. On one side a 0.9-cm hole is located to allow the mice to grab for food pellets (Dustless precision pellets, 14 mg; Plexx) with

only one paw at a time. After a fasting period of 14 h, mice were placed in the Collins apparatus and a total number of 20 grabbing attempts were observed. The Collins score was determined as follows: grabbing $attempt_{contralateral\ paw}$ / (grabbing $attempt_{contralateral\ paw}$ + grabbing $attempt_{ipsilateral\ paw}$).

Statistics

We used GraphPad Prism version 7.00 for Mac OS X, GraphPad Software, San Diego, California, USA, www.graphpad.com for all statistical analysis. Since there were no differences between control transplantations that were matched with different genetically-modified transplants, control transplantations were joined together in one group for comparisons. The distribution of data was analyzed by Kolmogorov-Smirnov, d'Agostino and Shapiro-Wilk tests. Normally distributed data were analyzed using the Student's t-test (for 2 groups) or by 1-way ANOVA test (for more than 2 groups) with Sidak's or Tukey's post-hoc tests that included correction for multiple comparisons. Non-normally distributed data were analyzed using the Kruskall-Wallis test with Dunn's post-hoc tests. For simultaneous comparison of 2 parameters between 2 and more groups, we used the 2-way-ANOVA and the Bonferroni post-hoc test. Equality of variances was analyzed using the Bartlett's test.

Author contributions

KK and MS conceptualized and supervised the study

KK, MS, IK, SS, DG, UP, AK, IS and AB designed, performed experiments and analyzed data

KK, MS, IK and SS wrote the manuscript

OD analyzed the infarct zone

All authors edited the manuscript

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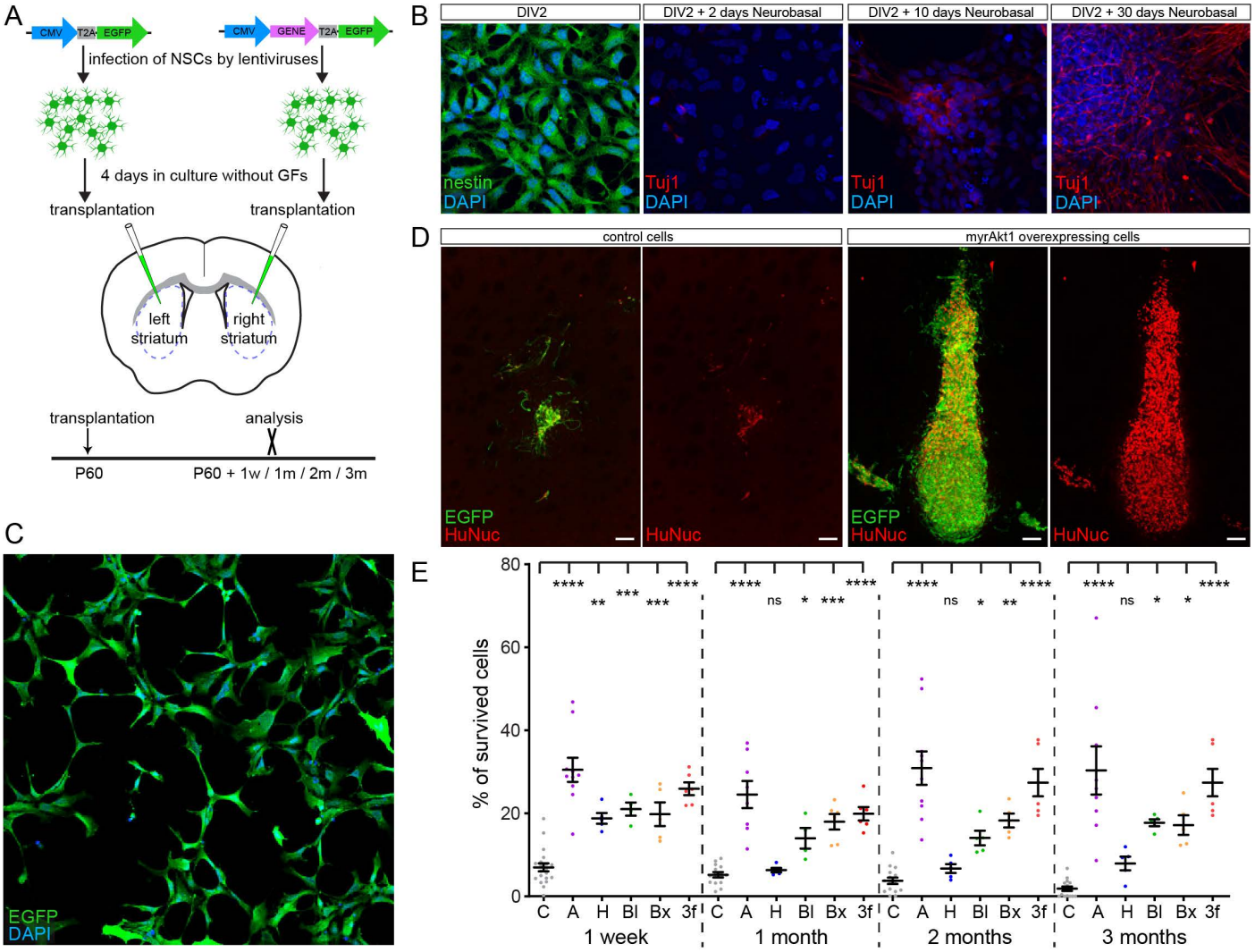
Figure 1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T2A
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CMV
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T2A
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infection of NSCs by lentiviruses
4 days in culture without GFs
transplantation
transplantation
left striatum
right striatum
transplantation
analysis
P60
P60 + 1w / 1m / 2m / 3m
B
DIV2
DIV2 + 2 days Neurobasal
DIV2 + 10 days Neurobasal
DIV2 + 30 days Neurobasal
nestin DAPI
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control cells
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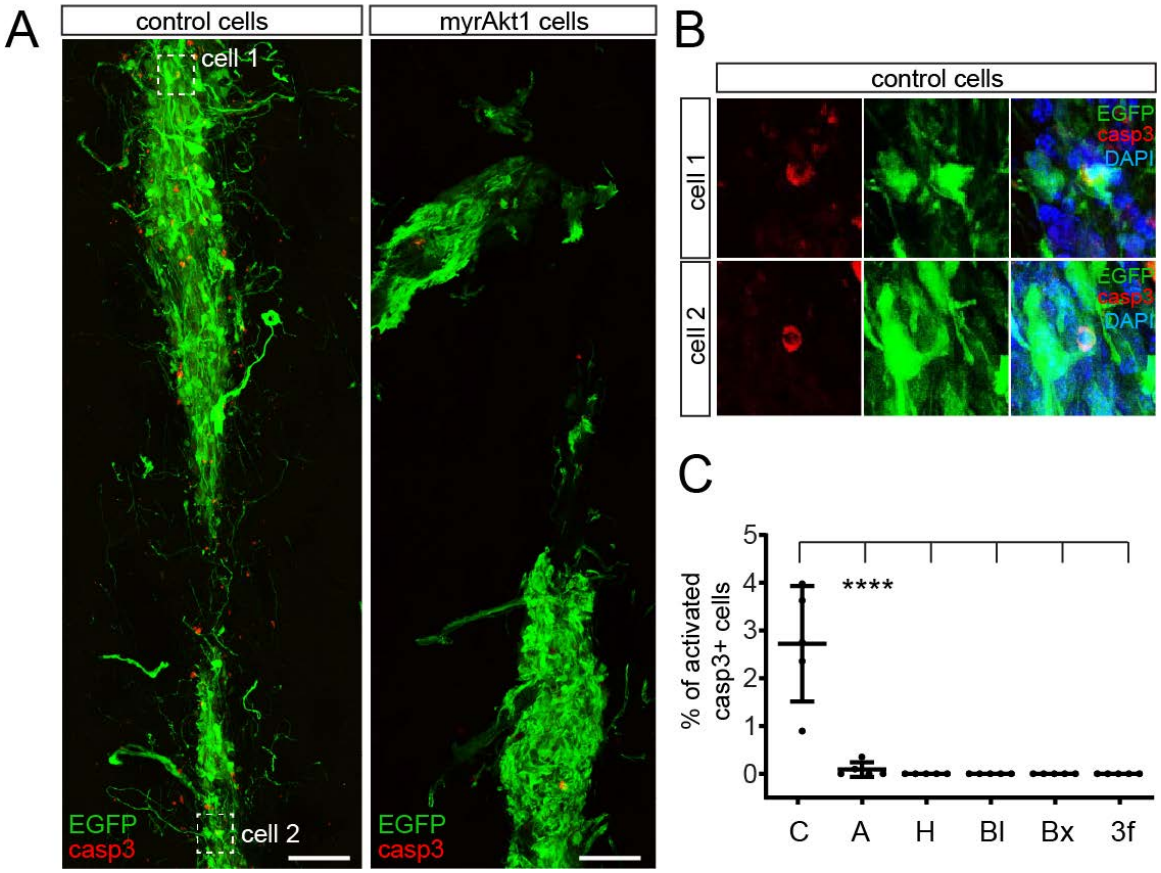
Figure 1. Genetic modification of H9 hNSCs strongly enhances their survival after transplantation into the striatum. (A) Cultured H9 hNSCs were infected by pCDH-CMV-MCS-T2A-EGFP lentivirus, empty or expressing *Akt1*, *Hif1a*, *Bcl2* or *Bcl2l1* genes. Cells were incubated 4 days in the medium without growth factors and then transplanted into the striatum of 60 days old NSG mice: control cells into the left and genetically-modified cells into the right striatum, respectively. Transplants were analyzed 1 week, 1, 2 and 3 months post-transplantation. (B) Differentiation of H9 hNSCs into neurons in vitro. Nestin (neuronal stem cell/precursor marker) and Tuj1 (neuronal marker) staining of H9 hNSCs at different timepoints of cell culture: DIV2 (day in vitro 2) without neurobasal medium; DIV2 +2, +10 and +30 days in the presence of neurobasal medium. (C) H9 hNSCs infected by pCDH-CMV-MCS-T2A-EGFP lentivirus, 4 days post-infection. (D) Control and myrAkt1-overexpressing cells H9 hNSCs 1 month post-transplantation. (E) Estimation of H9 hNSCs survival (% of total transplanted cells): 1 week, 1, 2 and 3 months after transplantation (n controls 14-20, n genetically-modified 5-10, for each time-point). C - empty vector, A - *Akt1*, H - *Hif1a*, Bl - *Bcl2*, Bx – *Bcl2l1*, 3f – *Akt1* + *Bcl2* + *Bcl2l1* expressing cells. Mean with SEM, 1-way ANOVA with Sidak's post-hoc tests, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$. Scale bars: in (D) - 100 µm.

Figure 2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control cells
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cell 1
cell 2
EGFP
casp3
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casp3
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control cells
cell 1
cell 2
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EGFP
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C
% of activated casp3+ cells
5
4
3
2
1
0
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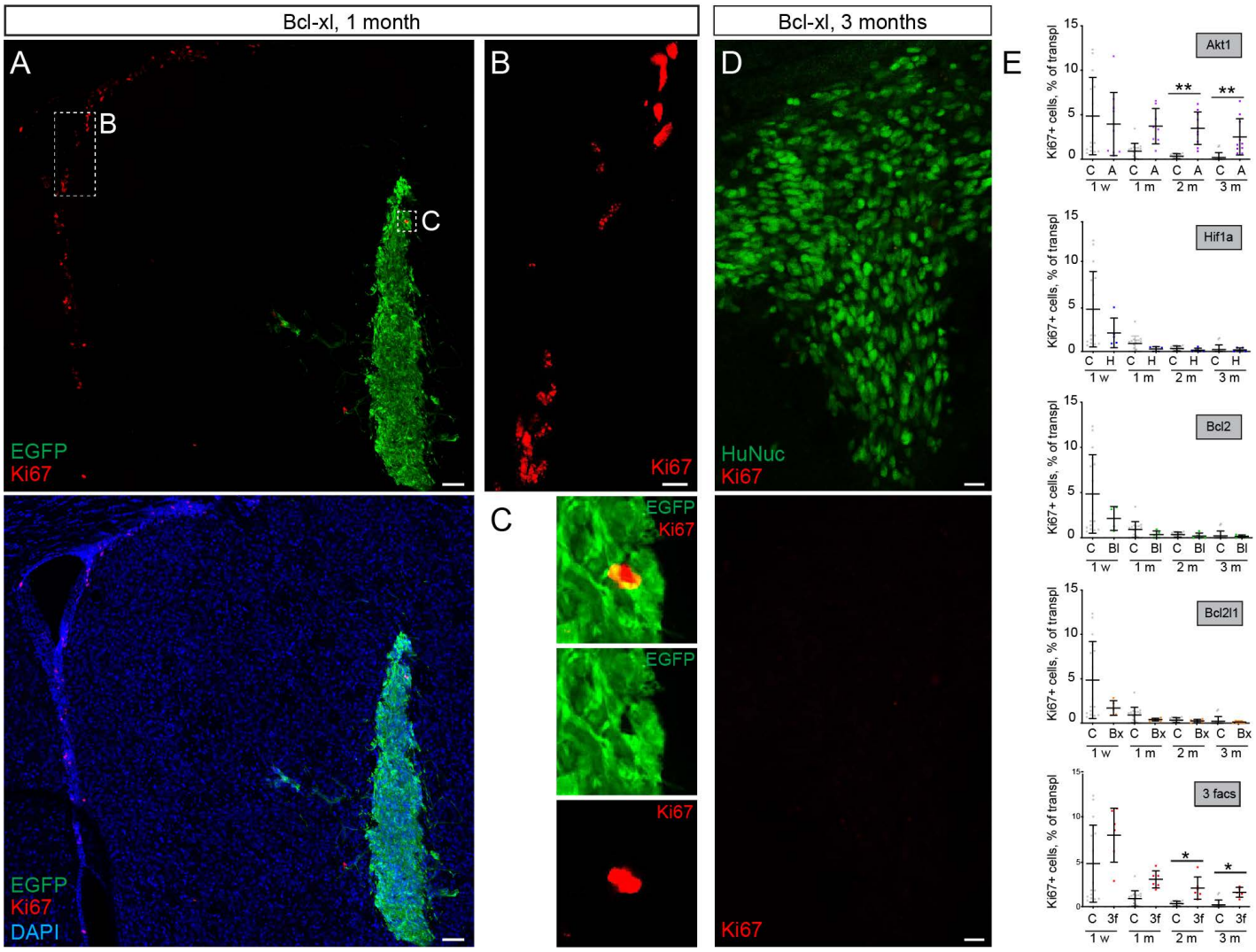
C
A
H
Bl
Bx
3f

Figure 2. Genetic modification of H9 hNSCs abrogates their cell death in vivo. (A) Control (left) and *Akt1*-expressing (right) cells (5 days post-transplantation). (B) Magnified images of control cells, co-expressing EGFP and activated caspase-3. (C) Percentage of transplanted cells expressing activated caspase-3 (n controls 5, n genetically-modified 5, for each time-point). Note that for H, Bl, Bx and 3f conditions we did not find any cells expressing activated caspase-3. C - empty vector, A - *Akt1*, H - *Hif1a*, Bl - *Bcl2*, Bx – *Bcl2l1*, 3f – *Akt1* + *Bcl2* + *Bcl2l1* expressing cells.

Mean with SD, Kruskal-Wallis with Dunn's post-hoc tests, **** - $p < 0.0001$.

Scale bars: in (A) – 100 μm.

Figure 3


Bcl-xl, 1 month
Bcl-xl, 3 months
A
B
C
D
E
EGFP
Ki67
DAPI
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Akt1
Hif1a
Bcl2
Bcl2l1
3 facs
Ki67+ cells, % of transpl
1 w
1 m
2 m
3 m

Figure 3. Genetically modified H9 hNSCs exit cells cycle soon after transplantations. (A – C) Transplanted *Bcl2l1* expressing H9 hNSCs 1 month post-transplantation stained with cell cycle marker Ki-67. Note that while Ki67 robustly labels postnatal neurogenesis in the subventricular zone (B), very few cells in the transplant are labeled by Ki67, and those are often not EGFP-positive thus representing rather endogenous glial cells rather than transplanted cells. (D) Transplanted *Bcl2l1* expressing cells 3 months post-transplantation. (E) Percentage of Ki67-positive cells in transplanted cells across different conditions 1 week, 1, 2 and 3 months after transplantation (n controls 14-16, n genetically-modified 5-10, for each time-point). C - empty vector, A - *Akt1*, H - *Hif1a*, Bl - *Bcl2*, Bx – *Bcl2l1*, 3f – *Akt1* + *Bcl2* + *Bcl2l1* expressing cells. Mean with SD, Kruskal-Wallis with Dunn's post-hoc tests, only significant values are shown, * - $p < 0.05$, ** - $p < 0.01$. Scale bars: in (A) - 100 μm, in (B) and (D) – 20 μm.

Figure 4

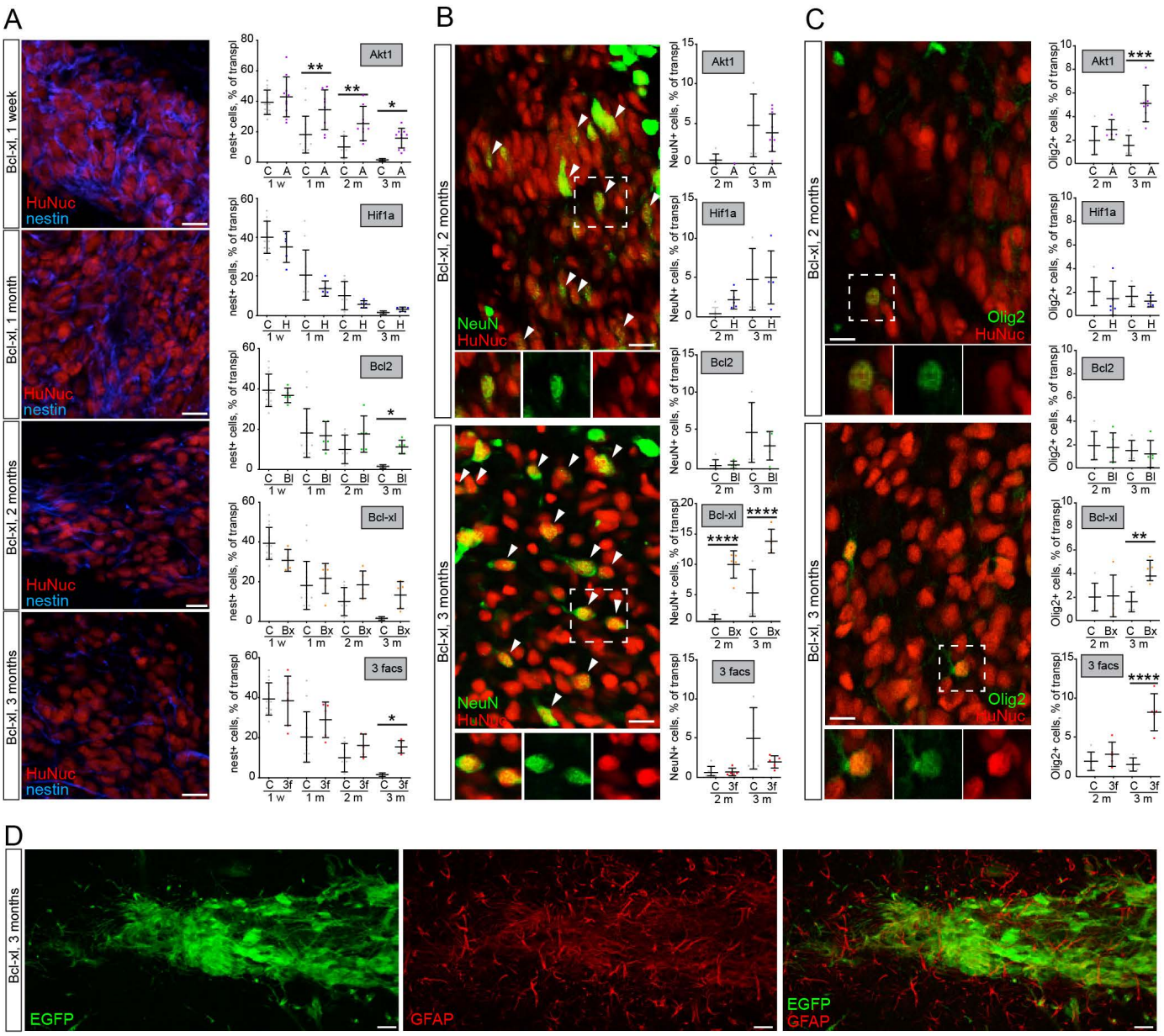
A
Bcl-xl, 1 week
Bcl-xl, 1 month
Bcl-xl, 2 months
Bcl-xl, 3 months
HuNuc
nestin
nest+ cells, % of transpl
Akt1
Hif1a
Bcl2
Bcl-xl
3 facs
C A 1 w 1 m 2 m 3 m
C H 1 w 1 m 2 m 3 m
C Bl 1 w 1 m 2 m 3 m
C Bx 1 w 1 m 2 m 3 m
C 3f 1 w 1 m 2 m 3 m
B
Bcl-xl, 2 months
Bcl-xl, 3 months
NeuN
HuNuc
NeuN+ cells, % of transpl
C A 2 m 3 m
C H 2 m 3 m
C Bl 2 m 3 m
C Bx 2 m 3 m
C 3f 2 m 3 m
C
Bcl-xl, 2 months
Bcl-xl, 3 months
Olig2
HuNuc
Olig2+ cells, % of transpl
D
Bcl-xl, 3 months
EGFP
GFAP

Figure 4. Transplanted genetically modified H9 hNSCs progressively differentiate into neural cells of neuronal and oligodendroglial fate. (A) Left panel – representative images of transplanted cells expressing *Bcl2l1* and stained with nestin, a marker of neural precursor cells. Right panel – percentage of nestin-positive cells in transplanted H9 hNSCs, 1 week, 1, 2 and 3 months after transplantation (n controls 5-12, n genetically-modified 5-10). (B) Left panel – representative images of transplanted cells expressing *Bcl2l1* and stained with NeuN, a marker of mature neurons. Right panel – percentage of NeuN-positive cells in transplanted H9 hNSCs, 2 and 3 months after transplantation (n controls 5-6, n genetically-modified 5-7). (C) Left panel – representative images of transplanted cells expressing *Bcl2l1* and stained with Olig2, a marker of oligodendrocytes. Right panel – percentage of Olig2-positive cells in transplanted cells, 2 and 3 months after transplantation (n controls 5-7, n genetically-modified 5-7, for each time-point). (D) Transplanted genetically-modified H9 hNSCs do not differentiate into astrocytic lineage in 3 months as evident by lack of co-expression of astrocytic marker GFAP. C - empty vector, A - *Akt1*, H - *Hif1a*, Bl - *Bcl2*, Bx – *Bcl2l1*, 3f – *Akt1* + *Bcl2* + *Bcl2l1* expressing cells. Mean with SD, 1-way ANOVA with Sidak's post-hoc tests, only significant values are shown, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$.

Scale bars: in (A), (B), (C), (D) – 20 μm.

Figure 5

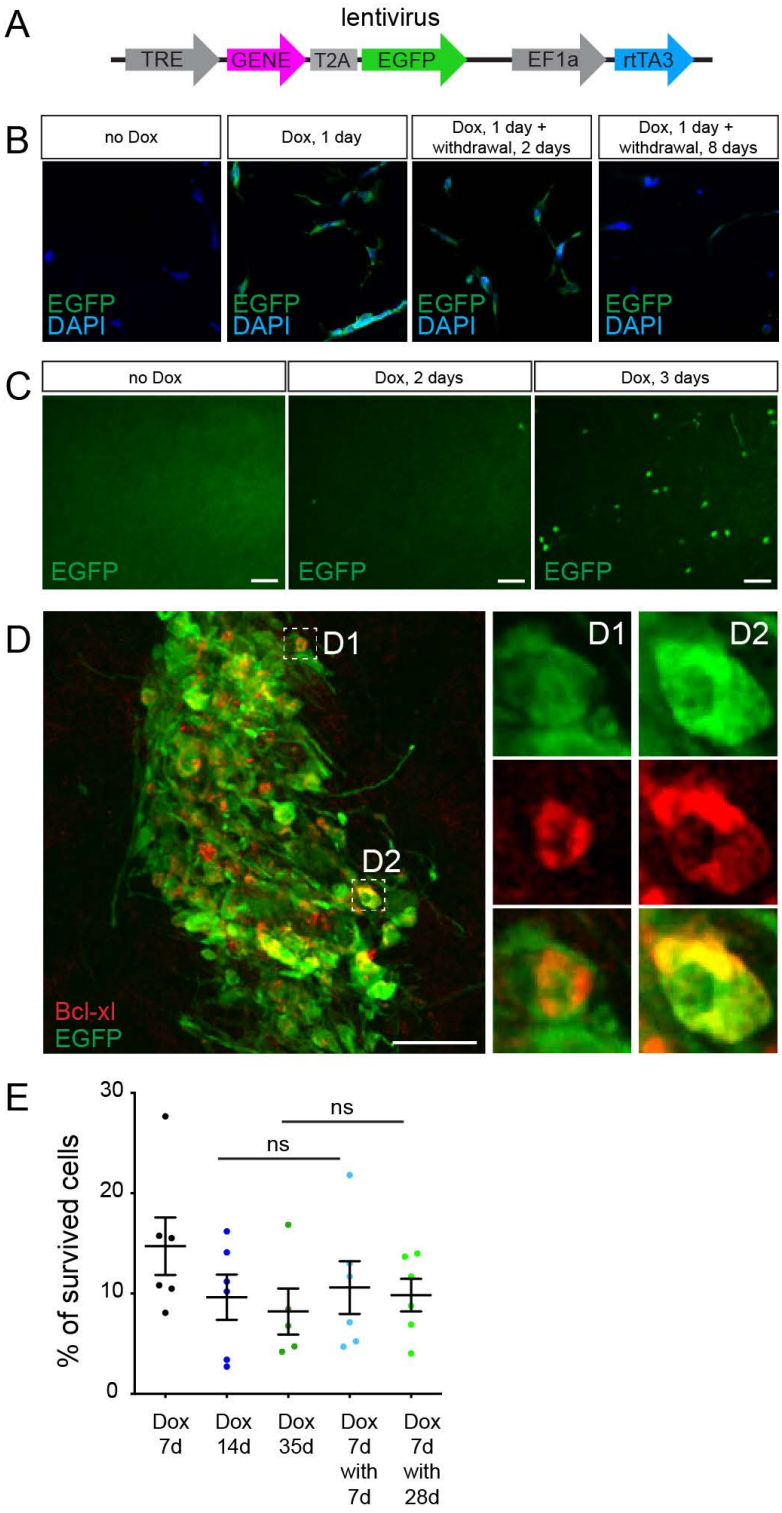
A
lentivirus
TRE
GENE
T2A
EGFP
EF1a
rtTA3
B
no Dox
Dox, 1 day
Dox, 1 day + withdrawal, 2 days
Dox, 1 day + withdrawal, 8 days
EGFP
DAPI
C
no Dox
Dox, 2 days
Dox, 3 days
EGFP
D
D1
D2
Bcl-xl
EGFP
E
% of survived cells
30
20
10
0
ns
ns
Dox 7d
Dox 14d
Dox 35d
Dox 7d with 7d
Dox 7d with 28d

Figure 5. Temporal activation of a pro-survival gene leads to long-term enhancement of survival of transplanted H9 hNSCs. (A) Lentiviral vector construct with doxycycline (Dox) inducible transgene expression. (B) H9 hNSCs infected with EGFP-coding lentiviral vector – without Dox, 1 day of Dox activation, 1 day of Dox activation following by 2 days of Dox withdrawal, 1 day of Dox activation following by 8 days of Dox withdrawal. (C) Dox-regulated EGFP-coding lentivirus was injected into the subventricular zone. Representative images of the olfactory bulb 20 days post-transplantation, when lentivirus-infected neuroblasts already migrated to the olfactory bulb. Mice were given Dox and EGFP-positive neurons in the olfactory bulb were labeled: before Dox was given to mice, 2 days and 3 days of Dox-activation. (D) H9 hNSCs were transduced by the lentivirus with Dox-activated expression of *Bcl2l1* and EGFP and co-express *Bcl2l1* and EGFP, 7 days post-transplantation. (E) Percentage of cell survival for H9 hNSCs that were transduced by the lentivirus with Dox-activated expression of *Bcl2l1* and EGFP and transplanted in the striatum (n 5-6); mice were treated with: Dox for 7 days post-transplantation (Dox 7d), Dox for 14 days (Dox 14d), Dox for 35 days (Dox 35d), Dox for 7 days followed by Dox withdrawal and 7 days of post-withdrawal (Dox 7d with 7d), Dox for 7 days followed by Dox withdrawal and 28 days of post-withdrawal (Dox 7d with 28d). Mean with SD, 1-way ANOVA with Sidak's post-hoc tests. Scale bars: in (C) and (D) – 50 μm.

Figure 6

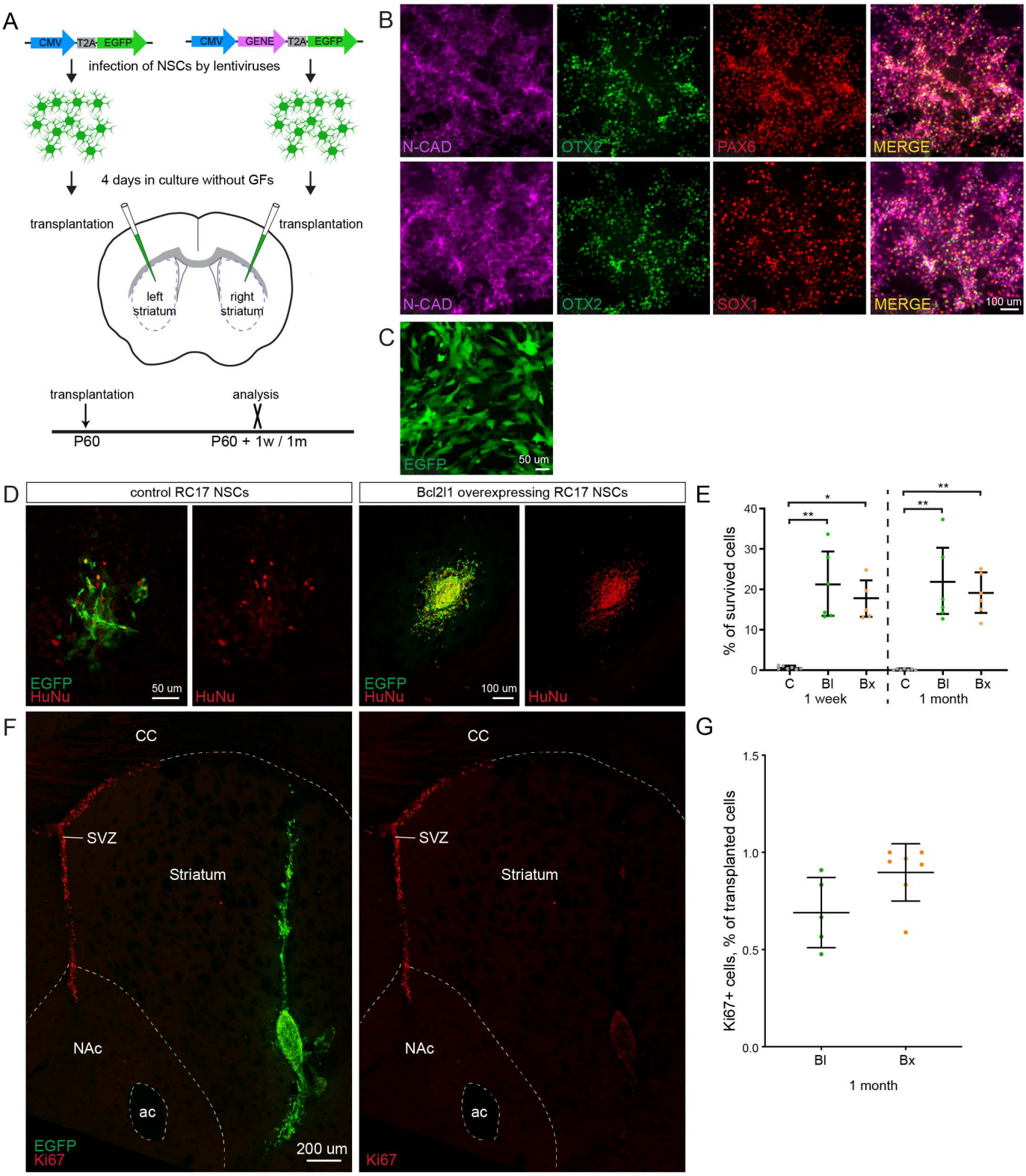
A
CMV
T2A
EGFP
CMV
GENE
T2A
EGFP
infection of NSCs by lentiviruses
4 days in culture without GFs
transplantation
transplantation
left striatum
right striatum
transplantation
analysis
P60
P60 + 1w / 1m
B
N-CAD
OTX2
PAX6
MERGE
N-CAD
OTX2
SOX1
MERGE
100 um
C
EGFP
50 um
D
control RC17 NSCs
Bcl2l1 overexpressing RC17 NSCs
EGFP
HuNu
50 um
HuNu
EGFP
HuNu
100 um
HuNu
E
% of survived cells
40
30
20
10
0
*
**
**
**
C
Bl
Bx
C
Bl
Bx
1 week
1 month
F
CC
SVZ
Striatum
NAc
ac
EGFP
Ki67
200 um
CC
SVZ
Striatum
NAc
ac
Ki67
G
Ki67+ cells, % of transplanted cells
1.5
1.0
0.5
0.0
Bl
Bx
1 month

Figure 6. Genetic modification of another hNSC line that is derived from RC17 ESCs reproduces pro-survival effect upon transplantation. (A) Cultured RC17 NSCs were infected by pCDH-CMV-MCS-T2A-EGFP lentivirus, empty or expressing *Bcl2* or *Bcl2l1* genes. Cells were incubated 4 days in the medium without growth factors and then transplanted into the striatum of 60 days old NSG mice: control cells into the left and genetically-modified cells into the right striatum, respectively. Transplants were analyzed 1 week and 1 month post-transplantation. (B) Differentiation of RC17 hESCs into NSCs was assessed by labeling of n-cadherin (neural progenitor marker), OTX2 (forebrain and midbrain marker), Pax6 (dorsal neural progenitor marker) and Sox1 (neural progenitor marker) staining of RC17 NSCs on day 15 in cell culture. (C) RC17 hNSCs infected by pCDH-CMV-MCS-T2A-EGFP lentivirus, 4 days post-infection. (D) Control and *Bcl2l1*-overexpressing RC17 hNSCs 1 month post-transplantation. Note different scale bars (larger scale bar for control RC17 hNSCs to show few survived cells). (E) Estimation of RC17 hNSC survival (% of total transplanted cells): 1 week and 1 month after transplantation (n controls 7, n genetically-modified 5-6, for each time-point). (F) Transplanted *Bcl2l1*-expressing RC17 NSCs 1 month post-transplantation stained with cell cycle marker Ki-67. While Ki67 robustly labels postnatal neurogenesis in the subventricular zone, very few cells in the transplant are labeled by Ki67. (G) Percentage of Ki67-positive cells in transplanted cells 1 month after transplantation (n controls 2, n genetically-modified 7). Note that naïve RC17 hNSCs have very low survival that often precludes reliable quantification due to too few (or lack of) survived cells. C - empty vector, Bl - *Bcl2*, Bx – *Bcl2l1*. Mean with SD, 1-way ANOVA with Dunn's post-hoc tests, * - $p < 0.05$, ** - $p < 0.01$. Scale bars: in (B) and (D) – 100 μm, in (C) – 50 μm, in (F) – 200 μm.

Figure 7

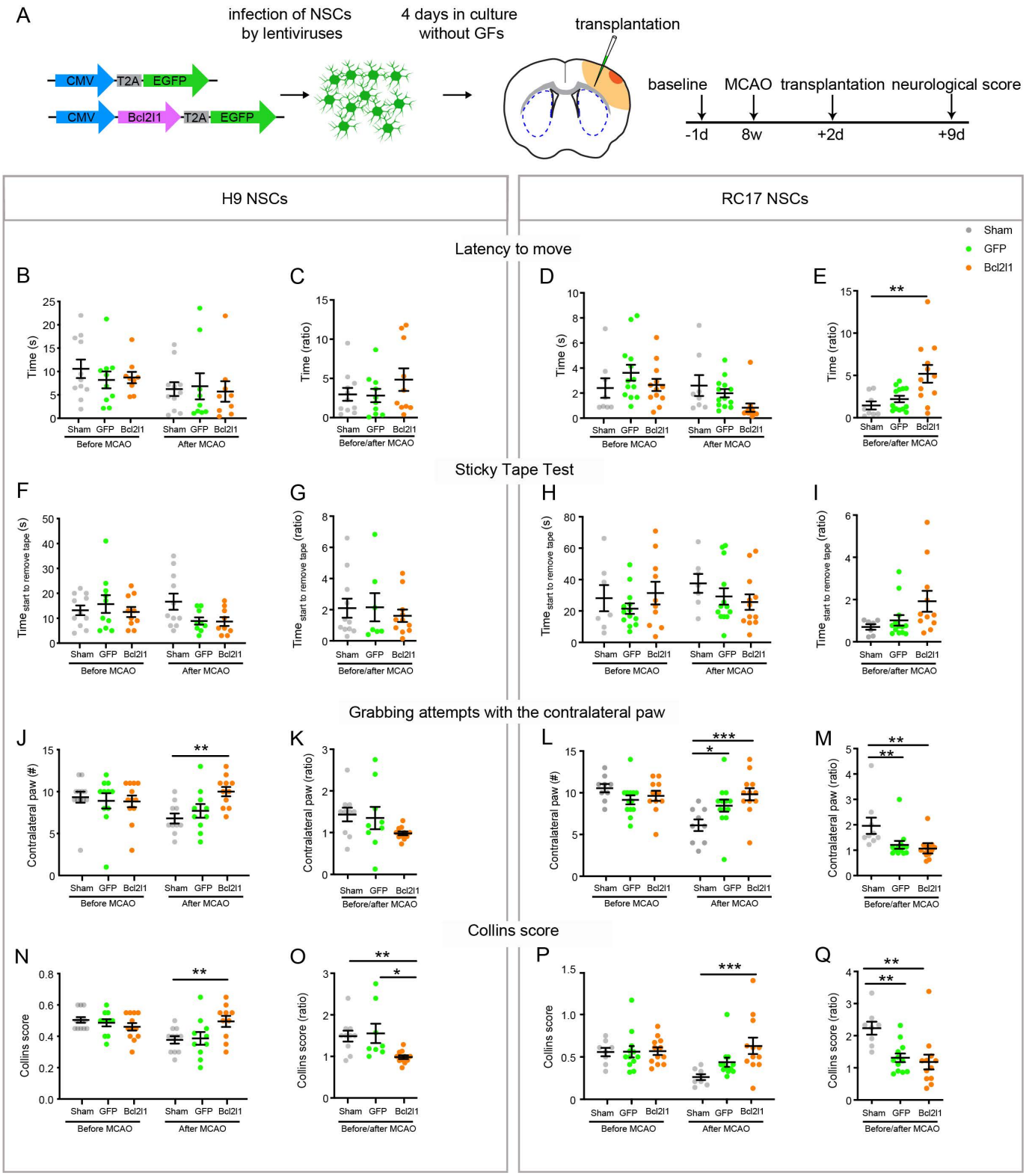
A
infection of NSCs by lentiviruses
4 days in culture without GFs
transplantation
CMV T2A EGFP
CMV Bcl2l1 T2A EGFP
baseline
MCAO
transplantation
neurological score
-1d
8w
+2d
+9d
H9 NSCs
RC17 NSCs
Sham
GFP
Bcl2l1
Latency to move
Sticky Tape Test
Grabbing attempts with the contralateral paw
Collins score
Time (s)
Time (ratio)
Time start to remove tape (s)
Time start to remove tape (ratio)
Contralateral paw (#)
Contralateral paw (ratio)
Collins score
Collins score (ratio)
Before MCAO
After MCAO
Before/after MCAO

Figure 7. Genetically-modified hNSCs improve ischemia-induced neurological deficits. (A) Two days after MCAO, EGFP only- or *Bcl2l1*-expressing H9 or RC17 NSCs were injected into the peri-infarct area and neurological score was estimated 7 days later. (B-E) Latency to move test. *Bcl2lI*-expressing RC17 NSCs significantly improved mobility in latency to move test compared to sham, indicated by the ratio before and after MCAO. (F-I) Stick tape test. No significant differences before and after MCAO as well as between sham, EGFP or *Bcl2l1* could be detected using sticky tape. (J-Q) The handiness of mice using Collins test. Transplantation of H9 and RC17 hNSCs with enhanced *Bcl2l1*-mediated survival increased the number of grabbing attempts with the contralateral paw compared to sham, thus improving behavior outcome to the level before MCAO (H9: J, 2-way ANOVA for interaction: $F_{(2/60)}$=3.51; RC17: L, 2-way ANOVA for interaction: $F_{(2/61)}$=6.22). RC17 hNSCs also significantly affected the ratio of grabbing attempts before and after MCAO (M). Both *Bcl2l1*-expressing H9 and RC17 hNSCs improved Collins score in comparison to sham-expressing hNSCs and H9 also in comparison to EGFP-expressing hNSCs, reaching a pre-MCAO level (H9: N, 2-way ANOVA for interaction: $F_{(2/60)}$=4.96; RC17: P, 2-way ANOVA for interaction $F_{(2/58)}$=3.41). The ratio of the Collins scores before and after MCAO was improved by transplantation of *Bcl2l1*-expressing hNSCs compared to sham- and, in case of H9, to EGFP-expressing hNSCs. For B, D, F, H, J, L, N and P: Mean with SEM, 2-way ANOVA, with Bonferroni posttest. For C, E, G,I , K, M, O and Q: Mean with SEM, 1-way ANOVA, Tukey's post-hoc test. * - $p < 0.05$, ** - $p < 0.01$, ***- $p < 0.001$; n=9-12.

Figure 8

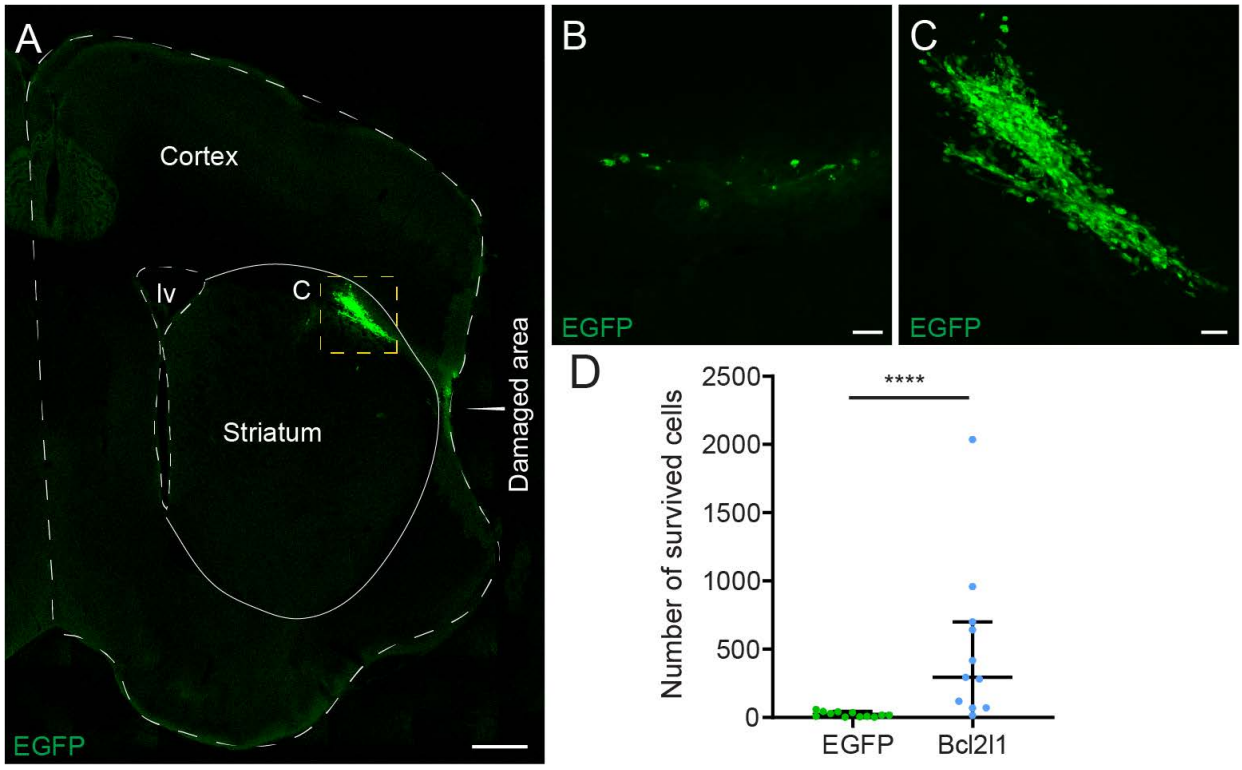
A
Cortex
lv
C
Striatum
Damaged area
EGFP
B
EGFP
C
EGFP
D
Number of survived cells
2500
2000
1500
1000
500
0
****
EGFP
Bcl2l1

Figure 8. *Bcl2l1*-overexpression strongly enhances the survival of hNSCs after transplantation into the mouse brain with ischemic injury. (A) Grafted genetically-modified RC17 hNSCs in the left hemisphere with ischemic injury (part of the brain is missing due to the massive cell death after MCAO, labeled by "Damaged area"). (B) Magnified images of control EGFP-expressing and *Bcl2l1*-expresing RC17 hNSCs. (D) Number of transplanted RC17 hNSCs 9 days after MCAO/ 7 days after transplantation. Median with IQR, Mann Whitney test. **** - $p < 0.0001$, n controls 11, n *Bcl2l1*-overexpresing 12.