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Brief Report

Reprogramming of Pericyte-Derived Cells of the Adult Human Brain into Induced Neuronal Cells

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SUMMARY

Reprogramming of somatic cells into neurons provides a new approach toward cell-based therapy of neurodegenerative diseases. A major challenge for the translation of neuronal reprogramming into therapy is whether the adult human brain contains cell populations amenable to direct somatic cell conversion. Here we show that cells from the adult human cerebral cortex expressing pericyte hallmarks can be reprogrammed into neuronal cells by retrovirus-mediated coexpression of the transcription factors Sox2 and Mash1. These induced neuronal cells acquire the ability of repetitive action potential firing and serve as synaptic targets for other neurons, indicating their capability of integrating into neural networks. Genetic fate-mapping in mice expressing an inducible Cre recombinase under the tissue-nonspecific alkaline phosphatase promoter corroborated the pericytic origin of the reprogrammed cells. Our results raise the possibility of functional conversion of endogenous cells in the adult human brain to induced neuronal fates.

Reprogramming of somatic cells into neurons provides a new approach toward cell-based therapy of neurodegenerative diseases (Vierbuchen and Wernig, 2011). Previous studies have shown that postnatal astroglia from the mouse cerebral cortex can be directly converted into functional neuronal cells in vitro by forced expression of a single transcription factor (Heinrich et al., 2010, 2011; Heins et al., 2002) and that the synergistic action of three or four transcription factors can induce neurogenesis from rodent and human fibroblasts (Caiazzo et al., 2011; Pang et al., 2011; Qiang et al., 2011; Son et al., 2011; Vierbuchen et al., 2010; Yoo et al., 2011). However, a major challenge for the translation of neuronal reprogramming into therapy is whether direct conversion of somatic cells into neuronal cells can be achieved from cells residing within the adult human brain. To address this question, we prepared adherent cultures from 30 human specimens that were derived from surgical approaches through the cerebral cortex to deep-seated nontraumatic nonmalignant lesions, i.e., epileptic foci and nonruptured vascular lesions. In order to characterize the cellular composition of the cultures obtained from these specimens, we performed immunocytochemistry and fluorescence-activated cell sorting (FACS) analyses at different stages of culturing. Intriguingly, the majority of cells expressed platelet-derived growth factor receptor-β (PDGFRβ) (Daneman et al., 2010) (Figures 1C and 1D and Figure S1A available online), which is detected within the human brain tissue exclusively on microvessel-associated pericytes (Figure 1A), a cell type involved in the establishment and maintenance of the blood-brain barrier and regulation of local blood flow (Armulik et al., 2011). Consistent with a pericyte identity, we also observed expression of NG2 (Karram et al., 2005) (Figure 1B and S1B), smooth muscle actin (SMA) (Figures S1A and S1B) (Hellström et al., 1999), CD146 (Crisan et al., 2008), and CD13 (Crisan et al., 2008) (Figure 1E), though with some heterogeneity with regard to coexpression of these markers (Figures 1E, S1A, and SB). In contrast, the number of glial acidic fibrillary protein (GFAP)-positive cells was extremely low in these cultures (<1%), although astrocytes were readily detected within the human tissue (data not shown). Quantitative RT-PCR experiments confirmed the enriched expression of pericytic marker genes and the virtual absence of astroglial (*gfap*)

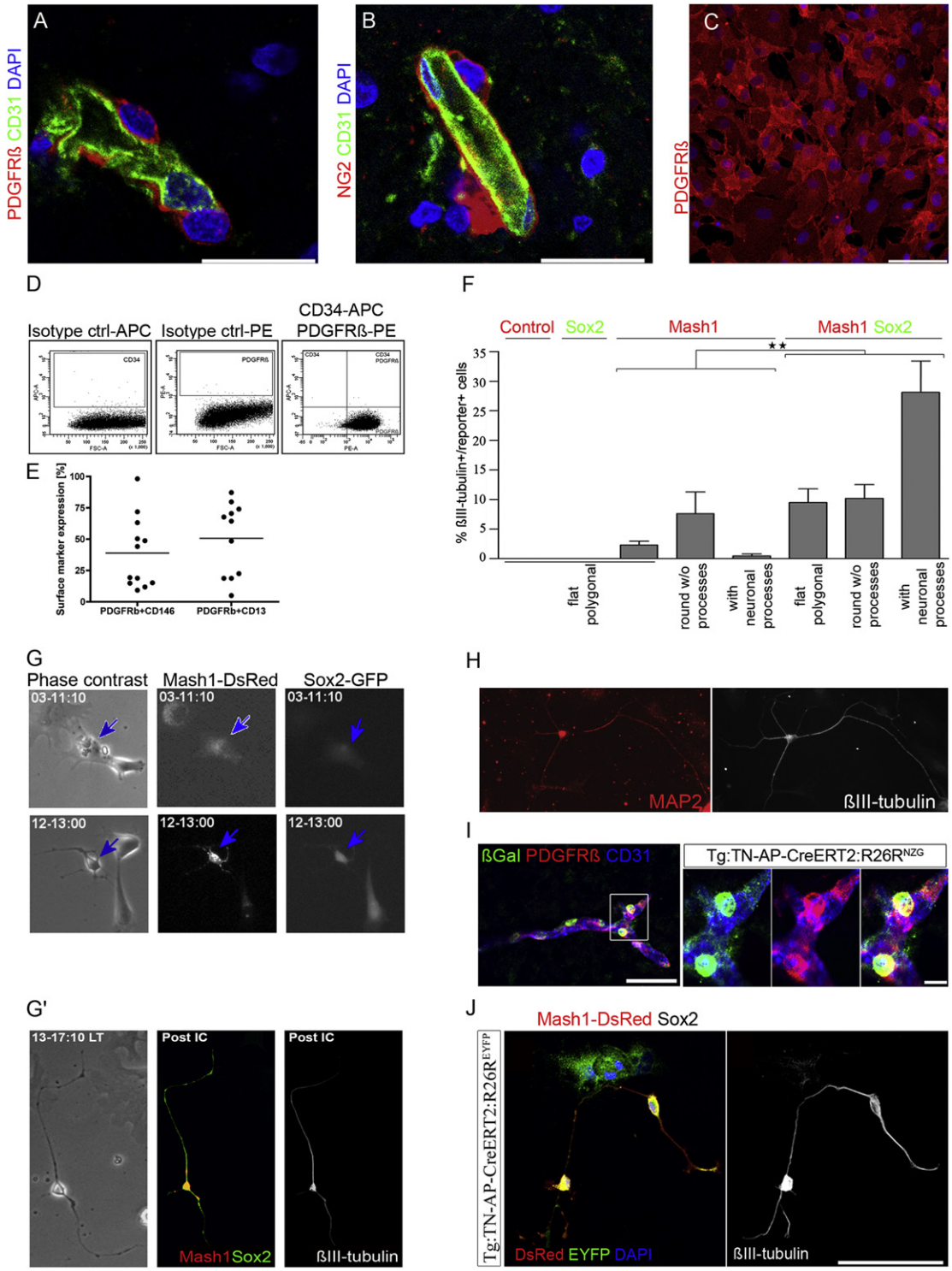

Figure 1. Characterization and In Vitro Conversion into Induced Neuronal Cells of Human and Mouse Adult Brain Pericyte-like Cells

(A) PDGFRβ expression in microvessel-associated cells in the adult human cerebral cortex. Scale bar: 100 μm.

(B) NG2 expression in microvessel-associated cells in the adult human cerebral cortex. Microvessels were visualized by CD31 (green) immunoreactivity and DAPI (blue). Scale bar: 100 μm.

(C) Immunocytochemical analysis for pericyte marker PDGFRβ (red) in cell cultures obtained from human cerebral tissue; DAPI is in blue. Scale bar: 100 μm. See also Figures S1A and S1D. Scale bar: 100 μm.

(D) Example of FACS analysis from an adult human brain culture. Depicted are the isotype controls (ctrl, left and middle panel) for establishing the gating conditions for sorting the PDGFRβ- and CD34-positive populations. See also Figure S1I.

and oligodendroglial cells (*olig2*) in these cultures compared to human brain tissue from which the cells had been isolated (Figure S1C). Importantly, βIII-tubulin could not be detected at any stage of culturing (assessed from 2 days to 8 weeks after plating), demonstrating that these cultures were devoid of neuroblasts or surviving neurons (data not shown). Furthermore, these cultures were completely devoid of expression of neural stem cell markers such as *sox2* or *prom1* or neurogenic fate determinants such as *ascl1* or *pax6* (Figure S1C). Moreover, *Sox2*, *Mash1*, Olig2, and *Pax6* were also not detected on the protein level by immunocytochemistry (data not shown). The few CD34-positive cells (Figures 1D and S1C) of hematopoietic or endothelial origin were lost upon passaging. Thus, these cultures are enriched for cells exhibiting pericyte characteristics.

Previous work has identified Mash1 (mammalian achaete-scute homolog 1, encoded by the gene *ascl1*) as a powerful reprogramming factor for direct conversion of somatic cells into neuronal cells (Berninger et al., 2007; Caiazzo et al., 2011; Vierbuchen et al., 2010). When we assessed the response of our cultures to retrovirus-mediated expression of Mash1 (CAG-*ascl1*-IRES-*dsred*), we observed the reduction of PDGFRβ expression to 23% (n[cells] = 219), indicating a loss of pericyte-specific protein expression (Figure S1D). Moreover, a subset of Mash1-transduced cells responded with the induction of βIII-tubulin, suggesting some degree of neuronal respecification (Figure 1F). Previous work has suggested that Sox2 expression may facilitate neuronal reprogramming of postnatal astrocytes by neurogenic fate determinants (Heinrich et al., 2010). As there was no endogenous Sox2 expression in these cultures (Figure S1C), we hypothesized that forced expression of *sox2* may enhance the efficiency of neuronal reprogramming by Mash1. Expression of Sox2 (CAG-*sox2*-IRES-*gfp*) alone had no overt effect on βIII-tubulin expression (Figure 1F) or morphology of pericyte-like cells (Figure S1F). In contrast, coexpression of Sox2 and Mash1 significantly increased the proportion of βIII-tubulin-expressing cells to 48% ± 9% SEM (n[cells] = 1,500, analyzed after 4–5 weeks following transduction, cultures from six different patients; compared to 10% ± 4% SEM after Mash1 transduction alone, p = 0.0038, Figure 1F). Most strikingly, many of the double-transduced cells (28% ± 5% SEM) exhibited neuronal morphology (Figure S1F) and induced expression of MAP2 (46% ± 11% SEM, n[cells] = 296 from three different patients, analyzed after 5–6 weeks; Figures 1H and S1G) and NeuN (Figure S1H), indicating a high degree of reprogramming efficiency of cells from adult human tissue. Consistent with the acquisition of a neuronal phenotype and a loss of pericyte identity, Sox2- and Mash1-coexpressing cells downregulated PDGFRβ (Figure S1E). Of note, some cultures contained virtually only (97%) PDGFRβ-positive cells (Figure 1D), of which 46% of the Mash1 and Sox2 cotransduced cells differentiated into βIII-tubulin-positive cells, with 26% exhibiting neuronal morphology (n[cells] = 203). In the following we refer to these neuronal cells derived from human pericyte-like cells as human pericyte-derived induced neuronal cells (hPdiNs).

Despite the high frequency of PDGFRβ-positive cells infected by the retroviral vectors, the remainder of PDGFRβ-negative cells may still act as the main source of induced neuronal cells upon Mash1 and Sox2 transduction. Thus, we proceeded to follow the fate conversion of pericytes by live imaging (Rieger et al., 2009). Cultured cells were FACS-sorted for surface expression of PDGFRβ (Figure S1I), transduced 48 hr later with retroviral vectors encoding *sox2* and *ascl1*, and subsequently imaged by time-lapse video microscopy (Movie S1). Figure 1G shows an example of an anti-PDGFRβ FACS-sorted cell undergoing Sox2- and Mash1-induced neurogenesis. The cell acquired a polarized morphology within 12 days following transduction and could be shown to express βIII-tubulin at the end of the live imaging (Figure 1G′). Intriguingly, following the onset of reporter expression, this PDGFRβ-sorted cell did not undergo any cell division, providing evidence for direct conversion from an adult human nonneuronal somatic cell into an hPdiN. Likewise, only 1 of 36 (3%) Sox2- and Mash1-coexpressing cells that we followed over time underwent cell division, in sharp contrast to untransduced (n[cells] = 11/30; 36%], Mash1-only (n[cells] = 8/30; 26%), and Sox2-only transduced cells (n[cells] = 13/30; 46%), indicating that Sox2- and Mash1-induced reprogramming does not only not require cell division, but is accompanied by immediate cell cycle exit. Of all the tracked cells coexpressing Sox2 and Mash1, 36% endured cell death. This percentage was considerably higher than that of untransduced cells (3%) and Sox2-only transduced cells (7%). Of note, Mash1-only transduced cells also exhibited a higher rate of cell death (33%), suggesting that Mash1 or Sox2 and Mash1 coexpression can induce a catastrophic conflict of cell fates in

(E) Relative coexpression of pericyte markers as analyzed by FACS analysis. Each data point represents the relative coexpression of PDGFRβ and CD146 (mean 40.7% ± 28.1%) or CD13 (mean 46.4% ± 29.1%).

(F) Quantification of the effect on βIII-tubulin expression and morphology following DsRed only for control, Sox2, Mash1, and combined Sox2 and Mash1 expression. Cells were categorized for exhibiting a flat polygonal morphology, round morphology without processes, or neuronal morphology with processes. Each value represents the mean of βIII-tubulin-positive cells from six different patients. For each patient and treatment, at least three experimental replicates were analyzed. For each condition >1,000 cells were analyzed. Error bars are SEM.

(G) Live imaging of the conversion of a PDGFRβ-positive FACS-sorted cell (blue arrow, see also Figure S1I) into an induced neuronal cell following coexpression of Sox2 and Mash1. Pictures show phase contrast and fluorescence (Mash1-DsRed and Sox2-GFP) images at different time points (Days-Hours:Minutes) during the reprogramming process. Note the change of the cotransduced cell from a protoplasmic to a neuron-like morphology. See also Movie S1.

(G′) Depicted is the last recorded time point in phase contrast (LT) and the postimmunocytochemistry (Post IC) of the reprogrammed cell for GFP (green), DsRed (red), and βIII-tubulin (white).

(H) Example of MAP2 and βIII-tubulin coexpression after 5 weeks following transduction. See also Figure S1G.

(I) Specific β-galactosidase expression associated with CD31-positive blood vessels in the cerebral cortex of Tg:TN-AP-CreERT2:R26R^{NZG} mice. β-galactosidase-positive cells express the pericyte marker PDGFRβ. Note the restricted expression around microvessels. β-galactosidase, green; PDGFRβ, red; CD31, blue. Scale bars: left panel, 50 μm; right panels, 10 μm.

(J) Reprogramming of EYFP-positive cells isolated from the cerebral cortex of adult Tg:TN-AP-CreERT2:R26R^{EYFP} mice into induced neuronal cells. EYFP-positive cells (green) transduced with Mash1 (red) and Sox2 (without reporter) display a neuronal morphology and express βIII-tubulin; 14 days postinfection. Scale bar: 100 μm. For the efficiency of reprogramming of mouse pericytic cells, see Figures S1J–S1K.

pericyte-derived cells. Counting of βIII-tubulin-positive cells after imaging revealed that none of the Sox2-only cells (n[cells] > 300), 7% of Mash1-only (n[cells] = 88) cells, and 25% of double-positive cells (n[cells] = 786; two independent experiments) expressed βIII-tubulin. In an additional experiment, in which cells had been sorted simultaneously for PDGFRβ and CD146 and had been time-lapsed, a reprogramming efficiency of 37% was observed (n[cells] = 209). Combining all time-lapse experiments, the overall reprogramming efficiency was 19% of the coinfected cells, taking proliferation and cell death into account.

To unequivocally determine the origin of the reprogrammed cells from pericytes in vivo, we turned to genetic fate-mapping in mice. We took advantage of a transgenic mouse that expresses an inducible Cre recombinase (CreERT2) under control of the tissue-nonspecific alkaline phosphatase (TN-AP) promoter for genetic fate mapping of pericytes (Dellavalle et al., 2011). These mice were crossed to reporter lines (Tg:TN-AP-CreERT2:R26R^{NZG} and Tg:TN-AP-CreERT2:R26R^{EYFP}) to aid identification of cells of pericytic origin either by β-galactosidase or yellow fluorescent protein (YFP) immunoreactivity following tamoxifen-induced Cre-mediated excision of the stop cassette. As expected β-galactosidase expression was confined to microvessel-associated cells coexpressing PDGFRβ (Figure 1I) and NG2 (data not shown) (Dellavalle et al., 2011) in the cerebral cortex of young adult mice following induction at postnatal stages, indicating that the TN-AP promoter allows reliable fate-mapping of pericyte-derived cells in the adult brain. Next we prepared cultures from the adult cerebral cortex of Tg:TN-AP-CreERT2:R26R^{EYFP} mice under the same culture conditions as used for human samples. As in the adult cerebral cortex, reporter-positive cells coexpressed the pericytic markers PDGFRβ, NG2, and CD146 and could be expanded in vitro (data not shown). In contrast to control vector-transduced reporter-positive pericyte-derived cells (data not shown), Sox2- and Mash1-expressing cells gave rise to βIII-tubulin-positive PdiNs (Figure 1J). Neuronal reprogramming of wild-type mouse pericyte-derived cells occurred at an even higher frequency compared to adult human pericyte-derived cells: coexpression of Sox2 and Mash1 significantly increased the proportion of βIII-tubulin-positive cells to 92% ± 3% SEM (compared to 41% ± 10% SEM after Mash1 transduction alone, p = 0.0028) (Figure S1K), and most of the double-transduced cells (73% ± 7% SEM) exhibited neuronal morphology (Figure S1J) and were capable of repetitive action potential firing (Figure S2F and Table S1).

We next analyzed whether the hPdiNs expressing neuron-specific proteins also acquire the functional membrane properties of neurons. In Mash1 (n[cells] = 7) and Sox2 (n[cells] = 6) singly transduced cells, step-current injection failed to elicit any action potentials (Figures S2A, S2A′, S2B, and S2B′), indicating that neither transcription factor alone induces neuronal electrical properties. In sharp contrast, a substantial proportion of cells (71% of 17 cells tested, cultures from five different patients) coexpressing both factors responded typically with the generation of a single action potential that could be blocked by the sodium channel antagonist tetrodotoxin (TTX) (Figures S2C and S2C′). Moreover, in voltage-clamp these cells exhibited clearly discernible sodium (Figure S2C″) and potassium (data not shown) currents. However, these hPdiNs exhibited immature properties, as reflected by the relatively high input resistances, low action potential, and peak sodium current amplitudes, even after prolonged time in culture, consistent with the slow maturation of human neurons (Table S1). In order to further promote maturation and to investigate whether hPdiNs can integrate into a neuronal network, we cocultured hPdiNs with neurons from the mouse embryonic neocortex. Under these conditions hPdiNs exhibited a more complex morphology (Figures 2A, 2B, and 2E) and were capable of repetitive action potential firing (Figure 2C), although input resistances were still high (Table S1). Importantly, hPdiNs were found to receive functional glutamatergic input from cocultured neurons (4 out of 12 cells analyzed, Figures 2D–2D″), demonstrating that they express functional transmitter receptors, are capable of assembling a postsynaptic compartment, and can be recognized by other neurons as functional targets. Consistent with functional glutamatergic input, dendrites of hPdiNs were decorated with presynaptic terminals containing vesicular glutamate transporters (Figure 2F). Of note, hPdiNs exhibited immunoreactivity for the inhibitory neurotransmitter β-aminobutyric acid (GABA, 14/14 hPdiNs analyzed) (Figure S2D). Moreover, qRT-PCR showed the expression of the interneuron calcium binding protein *pvalb* (Figure S2E), pointing toward acquisition of an interneuron-like phenotype. In contrast, none of the Sox2 and Mash1 cotransduced cells expressed the glutamatergic lineage marker *tbr1* (T-box brain gene 1; data not shown) or *slc17a7* (encoding the vesicular glutamate transporter [vGluT]-1; Figure S2E). However, a definitive proof for a GABAergic interneuron-like identity awaits the demonstration of functional GABAergic transmission.

Here we provide evidence for high-efficiency reprogramming of pericyte-derived cells of the adult human cerebral cortex into induced neuronal cells by coexpression of only two transcription factors. The fact that only coexpressing cells convert into neuronal cells provides direct evidence for a cell-autonomous effect. Different scenarios may account for the synergism of these two transcription factors. Sox2 may facilitate Mash1-induced reprogramming by rendering the somatic genome more susceptible to the neurogenic activity exerted by Mash1. Alternatively, Sox2 may be required to directly interact with Mash1 on common target genes. While we can currently not discern between these two modes of action, the fact that Neurog2 failed to reprogram cells in culture from the adult human cerebral cortex (data not shown) argues partially against the first mechanism as the solely important one. Recent studies on the role of Mash1 and Neurog2 during cortical development suggest that these factors activate distinct programs in neural progenitors (Castro et al., 2011). Mash1 also has been found as a key transcription factor in the direct reprogramming of fibroblasts (Pang et al., 2011; Vierbuchen et al., 2010) and hepatocytes (Marro et al., 2011) where it synergizes with Brn2 and Myt1l. This may suggest that Mash1 acts as a core factor in direct neuronal reprogramming. Interestingly, we observed a very slight induction of endogenous *ascl1* mRNA expression (Figure S2E). It is noteworthy that, while fibroblasts coexpressing different combinations of transcription factors have been shown to give rise to induced neuronal cells of glutamatergic identity (Pang et al., 2011; Vierbuchen et al., 2010), dopaminergic (Caiazzo et al., 2011; Kim et al., 2011; Pfisterer et al., 2011)

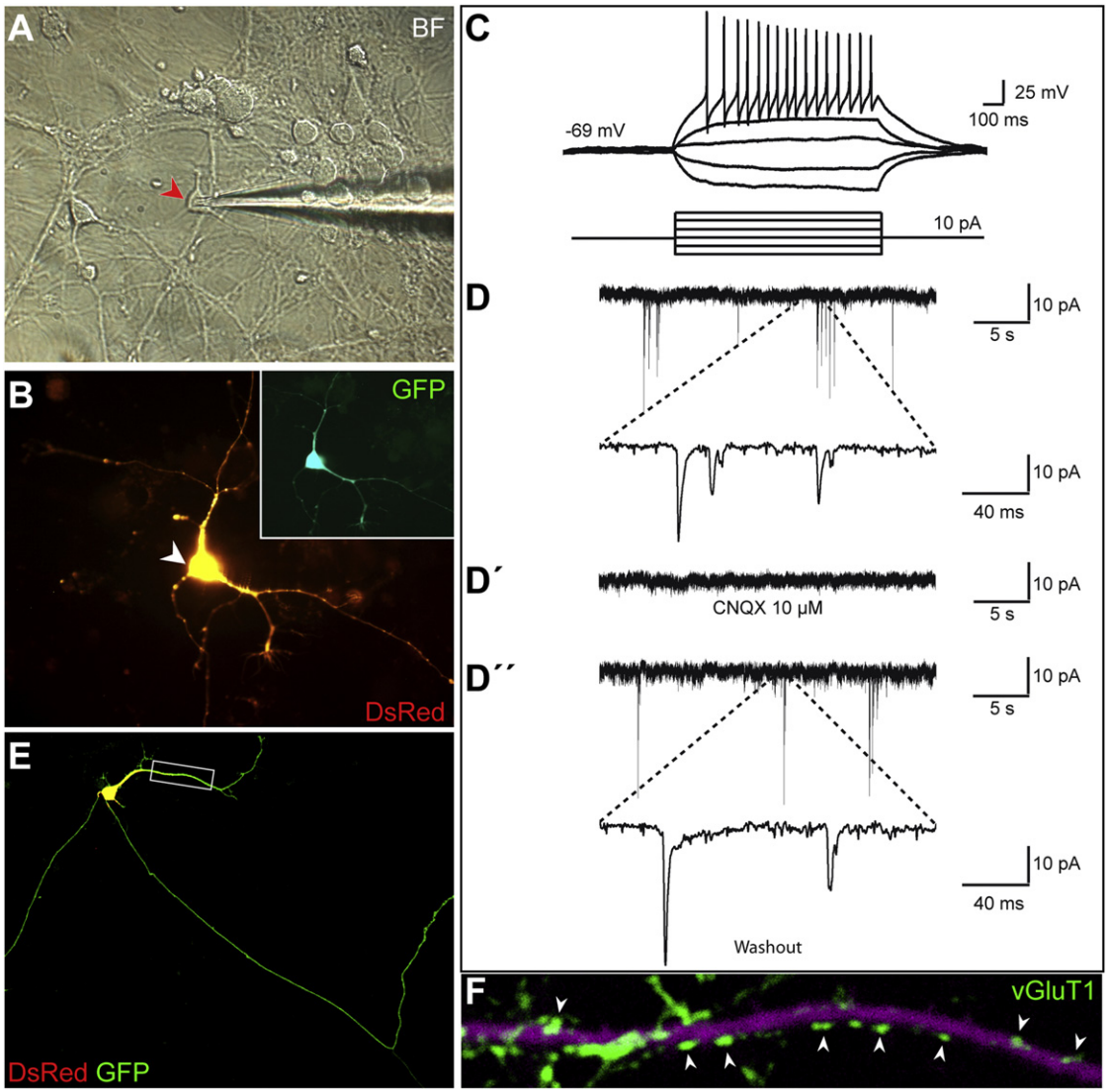

Figure 2. Neuronal Morphology and Membrane Properties of hPdiNs

(A) Bright-field micrograph depicts an hPdiN (red arrowhead) after 26 days of coculture with E14 mouse cerebral cortical neurons, 46 days following retroviral transduction.

(B) DsRed fluorescence indicating transduction with *ascl1* and *dsred*-encoding retroviruses. Inset: GFP fluorescence indicating transduction with *sox2*- and *gfp*-encoding retrovirus.

(C) Step current injection in current-clamp results in repetitive action potential firing. For comparison with cells transduced with a single transcription factor or cotransduced, but cultured without mouse cortical neurons, see Figures S2A–S2C″.

(D) The graph depicts spontaneous synaptic events recorded from the same hPdiN as shown in (C). The enlarged trace shows individual synaptic events.

(D′) The synaptic events are blocked by the application of CNQX (10 μM).

(D″) Recovery of spontaneous synaptic input following washout of CNQX. For a summary of the electrophysiological properties, see Table S1.

(E) Micrograph depicting an hPdiN stained for DsRed and GFP, after 22 days of coculture with E14 neurons, 42 days following retroviral transduction.

(F) High-magnification view of a single dendrite (magenta, GFP) from the same hPdiN as shown in (E), illustrating the high density and the distribution of vGluT1-immunoreactive puncta (green, Cy5).

and cholinergic motor neuron identity (Son et al., 2011), the combination of Sox2 and Mash1 appears to favor a GABAergic phenotype in hPdiNs. It will be important to understand whether this is largely dependent on the factor combination used or the cellular context determined by the origin and nature of the reprogrammed cell.

Local CNS pericytes have been recently recognized as a major source of proliferating scar-forming cells following CNS injury (Göritz et al., 2011). A key finding of the present study is that progeny of brain pericytes represent a potential target for direct reprogramming. While much needs to be learned about adapting a direct neuronal reprogramming strategy to meaningful repair in vivo, e.g., by using a noninvasive approach to activate these transcription factors (Kormann et al., 2011), our data provide strong support for the notion that neuronal reprogramming of cells of pericytic origin within the

damaged brain may become a viable approach to replace degenerated neurons.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes two figures, one table, Supplemental Experimental Procedures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.07.007.

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