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Abstract: Reprogramming of somatic cells into neurons provides a new approach towards cell-based therapy of neurodegenerative diseases. A major challenge for the translation of neuronal reprogramming into therapy concerns the question 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 co-expression 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 non-specific 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.

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Deborah Sweet, PhD
Editor, Cell Stem Cell

Revision of CELL-STEM-CELL-D-11-00764R2

Dear Deborah,

Thank you for your positive reply to our revision. We are happy with the modifications that you suggested and incorporated them accordingly. Furthermore, we adapted the graphical abstract to the requirements of the journal.
We look forward to your final decision.

Sincerely yours,

Benedikt Berninger, PhD

***Response to Reviewers**

We modified the manuscript according to the remaining concerns of the Reviewer#2 by replacing the term “neuron” by induced neuronal cells throughout the manuscript, as the Reviewer rightly pointed out that the term “neuron” should be reserved for real neurons isolated from the nervous system. Accordingly, we exchanged hPdNs by hPdiNs (human pericyte-derived induced neuronal cells).

Moreover, we also quantified the efficiency of reprogramming of pericyte-derived cells into MAP2-positive neuronal cells, analyzing double transduced cells from 3 different patients after 5-6 weeks of culture. The number is rather comparable to that of βIII tubulin expressing cells (approximately 45%, see page 6, line 1). We would like to point out that for MAP2 being expressed by these cells a rather long time of maturation is required, thus we could not perform the same live imaging analysis as for βIII tubulin, which is expressed much earlier during differentiation. However, with regard to the specificity, we would like to stress that none of the control or Sox2 singly- and very few of the Mash1 singly-transduced cells express βIII tubulin, arguing strongly against an unspecific expression. Finally, we included an example of MAP2 expression in the main Figure 1 (1H).

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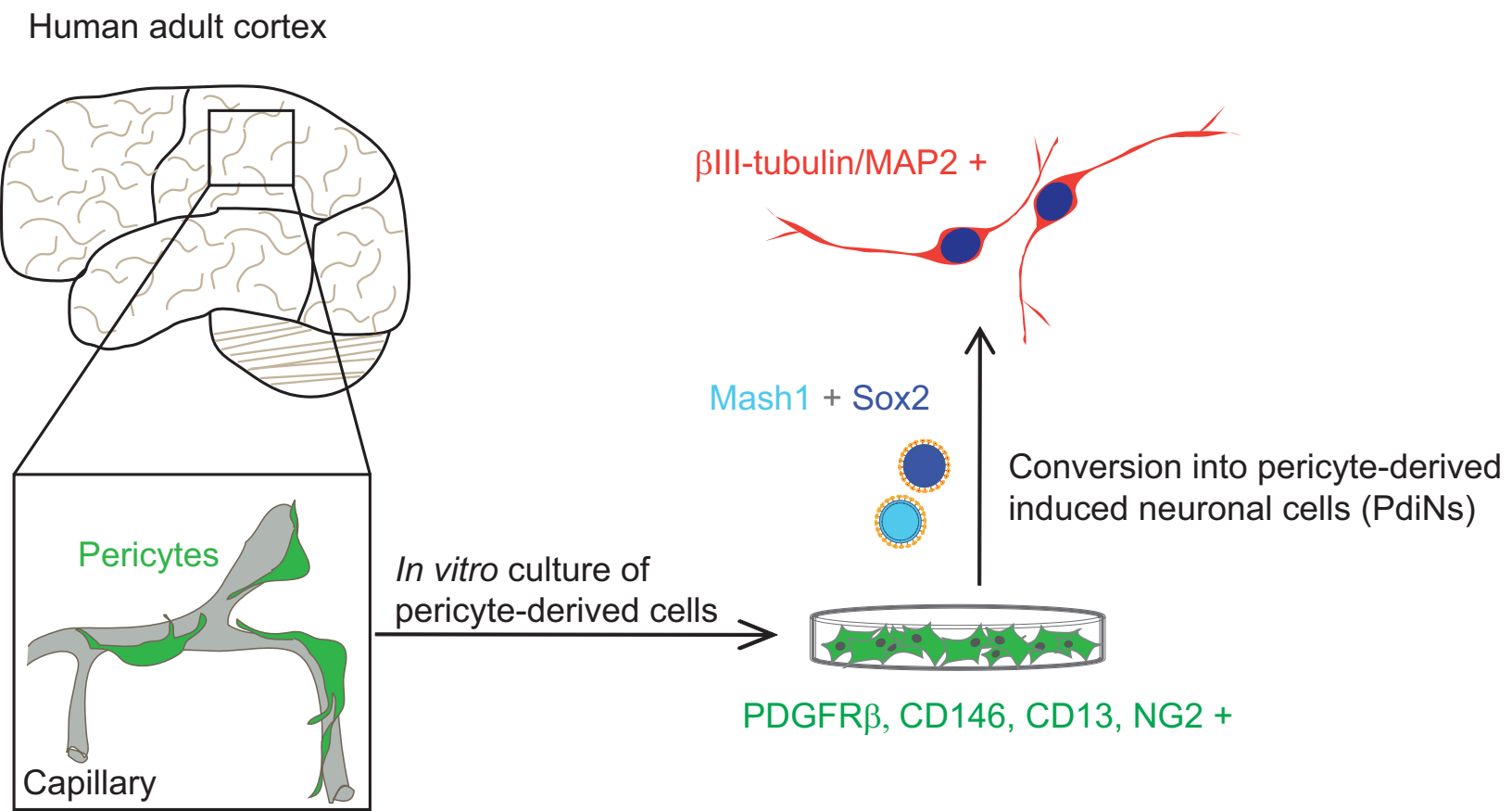

Highlights

- Neuronal reprogramming of adult human brain pericytes using Sox2 and Mash1
- Mouse genetic fate mapping confirms the pericyte origin of the converted cells
- Pericyte-derived induced neuronal cells (PdiNs) demonstrate neuronal excitability

Reprogramming of pericyte-derived cells of the adult human brain into induced neuronal cells

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Running Title

Two-factor pericyte-to neuron conversion

Summary

Reprogramming of somatic cells into neurons provides a new approach towards cell-based therapy of neurodegenerative diseases. A major challenge for the translation of neuronal reprogramming into therapy concerns the question 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 co-expression 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 non-specific 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.

Main text

Reprogramming of somatic cells into neurons provides a new approach towards 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; Heins et al., 2002) and the synergistic action of 3-4 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 concerns the

question 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 derived from surgical approaches through the cerebral cortex to deep-seated non-traumatic non-malignant lesions, i.e. epileptic foci and non-ruptured 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) (Figure 1C, D and S1A), 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) (Figure S1A and S1B) (Hellstrom et al., 1999), CD146 (Crisan et al., 2008) and CD13 (Crisan et al., 2008) (Figure 1E), though with some heterogeneity with regard to co-expression of these markers (Figure 1E, Figure S1A,B). 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) 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 CD133/Prominin1 or neurogenic fate determinants such as Mash1 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 (Figure 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 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-Mash1-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, suggestive of some degree of neuronal re-specification (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, co-expression of Sox2 and Mash1 significantly increased the proportion of βIII-tubulin-expressing cells to 48% ± 9 SEM (n[cells]=1500, analysed after 4-5 weeks following transduction, cultures from 6 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 3 different

patients, analysed after 5-6 weeks; Figure 1H, Figure 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 co-expressing cells down-regulated PDGFRβ (Figure S1E). Of note, some cultures contained virtually only (97%) PDGFRβ-positive cells (Figure 1D), of which 46% of the Mash1 and Sox2 co-transduced 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 hPdiNs (human pericyte-derived induced neuronal cells).

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. Cultured cells were FACS-sorted for surface expression of PDGFRβ (Figure S1I), transduced 48 hours later with retroviral vectors encoding Sox2 and Mash1 and subsequently imaged by time-lapse video microscopy (Video S1). Figure 1G shows an example of an anti-PDGFRβ FACS-sorted cell undergoing Sox2- and Mash1-induced neurogenesis. The cell acquired a polarised 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 non-neuronal somatic cell into an hPdiN. Likewise, only 1 of 36 (3%) Sox2- and Mash1-co-expressing cells 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 co-expressing 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-co-expression can induce a catastrophic conflict of cell fates in 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) and 25% of double-positive cells (n[cells]=786; 2 independent experiments) expressed βIII-tubulin. In an additional experiment, in which cells had been sorted simultaneously for PDGFRβ and CD146 and 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 co-infected 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 which expresses an inducible Cre recombinase (CreERT2) under control of the tissue non-specific 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 identify 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 co-expressing 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 for 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 co-expressed 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 wildtype mouse pericyte-derived cells occurred even at a higher frequency compared to adult human pericyte-derived cells: co-expression 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 analysed 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 (Figure S2A, A', B and B'), 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 5 different patients) co-expressing both factors responded typically with the generation of a single action potential which could be blocked by the sodium channel antagonist tetrodotoxin (TTX) (Figure S2C and C'). Moreover, in voltage-clamp these cells exhibited clearly discernible sodium (Figure S2C'') and potassium currents (data not shown). 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 co-cultured hPdiNs with neurons from the mouse embryonic neocortex. Under these conditions hPdiNs exhibited a more complex morphology (Figure 2A,B,E) 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 co-cultured neurons (4 out of 12 cells analysed, Figure 2D-D''), demonstrating that they express functional transmitter receptors, are capable of assembling a postsynaptic compartment and can be recognised 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 PdiNs analysed) (Figure S2D). Moreover qRT-PCR showed the expression of the interneuron calcium binding protein parvalbumin (Figure S2E) pointing towards acquisition of an interneuron-like phenotype. In contrast, none of the Sox2 and Mash1 co-transduced cells expressed the glutamatergic lineage marker Tbr1 (data not shown) or vGluT1 (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 co-expression of only two transcription factors. The fact that only co-expressing 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 Mash1 mRNA expression (Figure S2E). Noteworthy, while fibroblasts co-expressing 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) and cholinergic motor neuron identity (Son et al., 2011), the combination of Sox2 and Mash1 appears to favour 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 recognised as a major source of proliferating scar-forming cells following CNS injury (Goritz et al., 2011). A key finding of the present study is that progeny of brain pericytes represents a potential target for direct reprogramming. While much needs to be learnt about adapting a direct neuronal reprogramming strategy to meaningful repair in vivo, e.g. by using a non-invasive 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.

Figures

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. (B) NG2 expression in microvessel-associated cells in the adult human cerebral cortex. Microvessels were visualized by CD31 (green) immunoreactivity; DAPI (blue). Scale bars: 100 µm. (C) Immunocytochemical analysis for pericyte marker PDGFRβ (red) in cell cultures obtained from human cerebral tissue; DAPI (blue). Scale bar: 100µm. See also Figures S1A and S1D. (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 Figures S1I. (E) Relative co-expression of pericyte markers as analysed by FACS analysis. Each data point represents the relative co-expression of PDGFRβ and CD146 (mean 40.7% ± 28.1) or CD13 (mean 46.4% ± 29.1), respectively. (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, round morphology without processes, or neuronal morphology with processes. Each value represents the mean of βIII-tubulin-positive cells from 6 different patients. For each patient and treatment at least 3 experimental replicates were analysed. For each condition >1000 cells were analysed. 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 co-expression 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 co-transduced cell from a protoplasmic to neuron-like morphology. See also Supplemental Movie S1. (G') Depicted is the last recorded time point in phase contrast (LT) and the post-immunocytochemistry (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 DPI. Scale bar: 100 µm. For the efficiency of reprogramming of mouse pericytic cells see Figure S1J-K.

Figure 2. Neuronal morphology and membrane properties of hPdiNs. (A) Bright field micrograph depicts a hPdiN (red arrowhead) after 26 days of co-culture with E14 mouse cerebral cortical neurons, 46 days following retroviral transduction. (B) DsRed fluorescence indicating transduction with Mash1 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 co-transduced, but cultured without mouse cortical neurons see Figure 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 Supplemental Table S1. (E) Micrograph depicting a hPdiN stained for DsRed and GFP, after 22 days of co-culture 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).

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Figure 1

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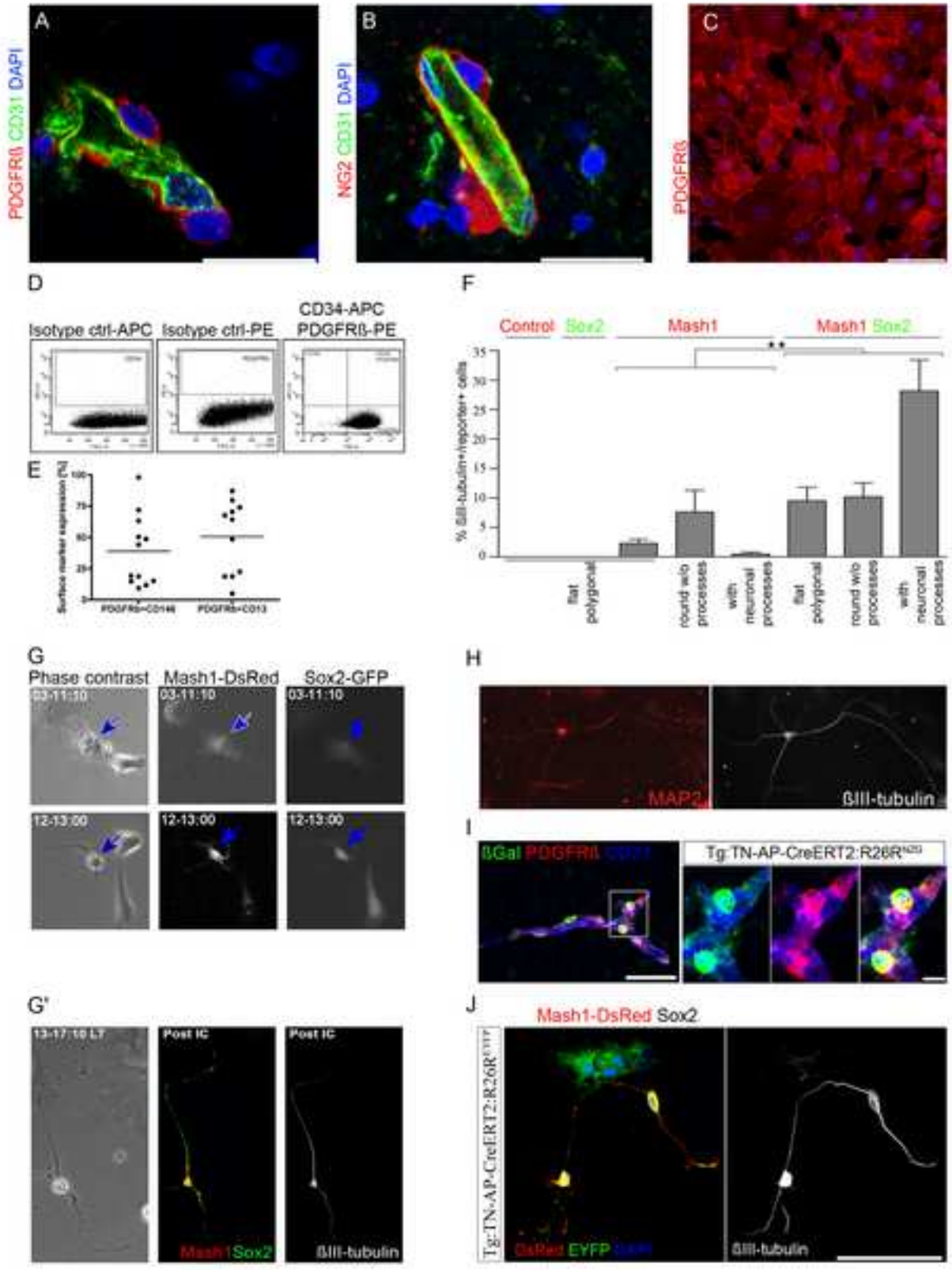

Figure 2

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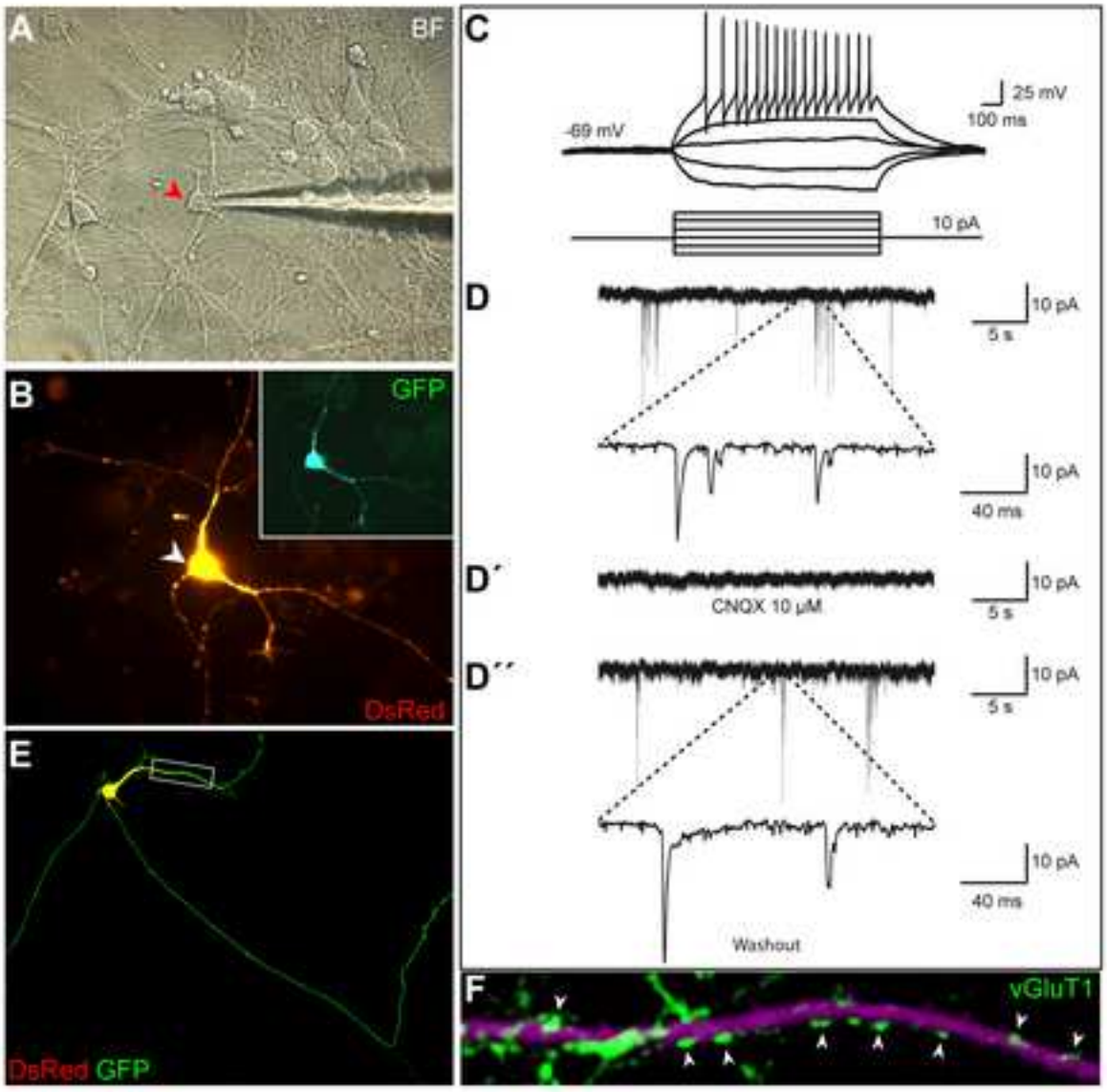

Inventory of supplemental items:

Figure S1: complements the characterisation of the pericytic origin of the cells isolated from the adult human cerebral cortex (related to Figure 1A-E), provides further evidence for the neuronal phenotype of the Sox2 and Mash1 reprogrammed cells (related to Figure 1F and 1H), and shows the reprogramming efficiency of pericytic cells isolated from the mouse brain (related to Figure 1I-J).

Figure S2: complements the physiological and subtype properties of human and murine PdiNs (related Figure 2).

Movie S1: relates to Figure 1G-G' and S1I.

Table S1: summarizes electrophysiological properties of PdiNs (related to Figures 2 and S2).

Supplemental Experimental Procedures

Supplemental References: References referred to in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Figure S1

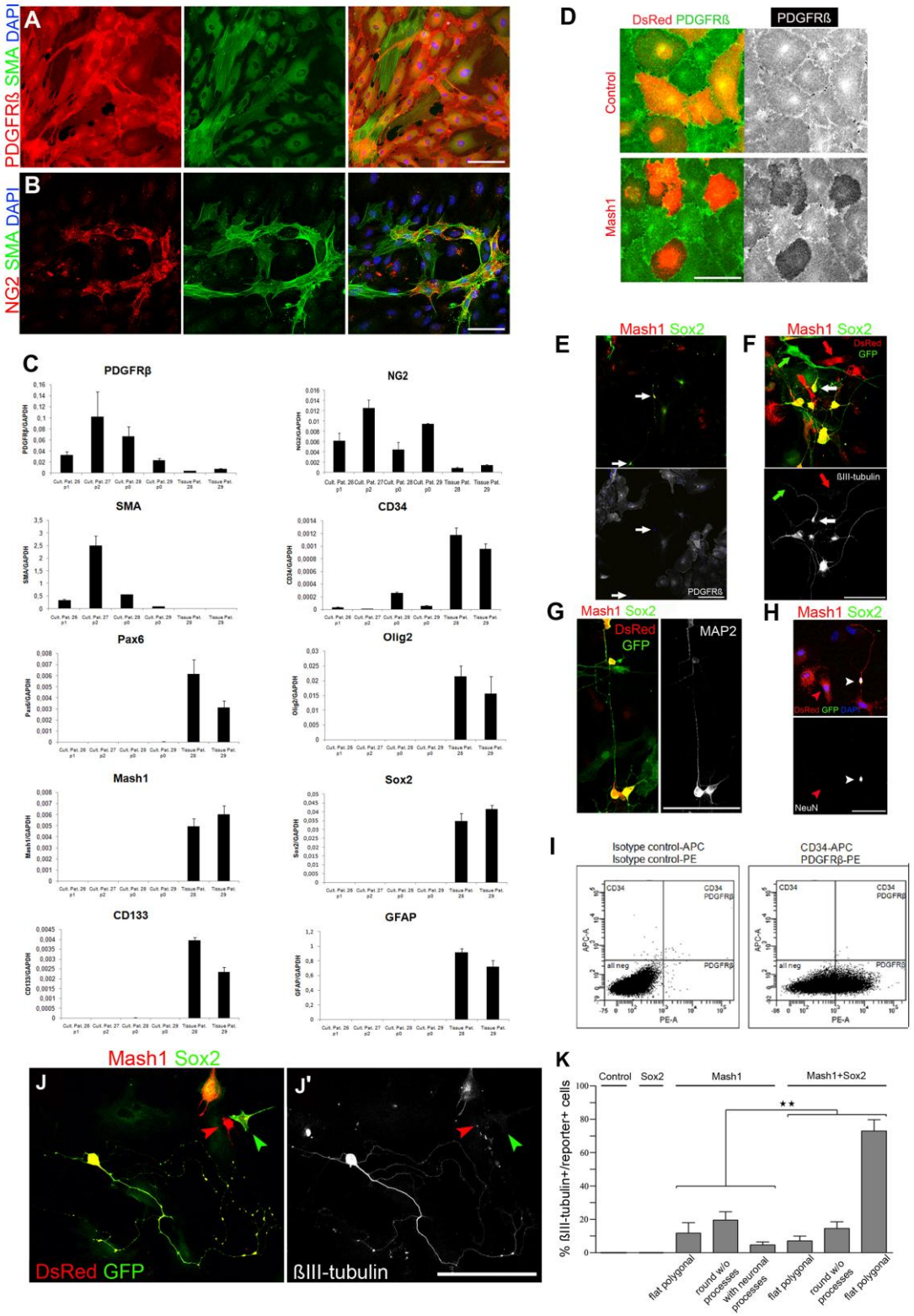

Figure S1. Corroboration of pericytic origin of Sox2 and Mash1 reprogrammed cells. (A) Immunocytochemical analysis for pericyte marker expression in cell cultures obtained from adult human cerebral cortical tissue. PDGFRβ (red), SMA (green), DAPI (blue): see also Figure 1C. (B) NG2 (red), SMA (green), DAPI (blue). Scale bars: 100µm**.** (C) Gene expression analyses in adult human cerebral cortical cultures. Quantitative RT-PCR analyses for expression of various mRNAs in cultures derived from different patients and different passages. Expression was normalized to mRNA levels of GAPDH and compared to the expression within the human tissue from which cells were isolated. Messenger RNAs analysed include PDGFRβ, NG2 (gene name Cspg4), α-smooth muscle actin (SMA), CD34, Pax6, Olig2, Mash1, Sox2, CD133, GFAP. Note the enrichment of the pericytic gene expression in cultures compared to the original tissue. (D) Down-regulation of PDGFRβ after forced expression of Mash1; upper panels control virus; lower panels Mash1-coding virus. Note the specific down-regulation of PDGFRβ expression after forced Mash1 expression, but not in controls, 22 days post infection. Scale bar: 50 µm. (E) Down-regulation of PDGFRβ (white) in PDGFRβ-sorted cells following co-expression of Sox2 (green) and Mash1 (red), 3 weeks after transduction. White arrows indicate co-transduced cells. DAPI (blue). Scale bars: 100µm**.** (F) Induction of a neuronal phenotype following co-expression of Sox2 and Mash1 (white arrow) in cultures from the adult human cerebral cortex. Note that cells expressing only Mash1 (red arrow) or only Sox2 (green arrow) are devoid of βIII-tubulin (white). Scale bar: 100 µm. (G) Cells transduced with Sox2 (green) and Mash1 (red) express the neuronal marker MAP2 (white). Scale bar: 100 µm. See also Figure 1H. (H) Cells transduced with Mash1 and Sox2 (white arrowhead) express the neuronal marker NeuN (white). Note the NeuN-negative Mash1-only expressing cell (red arrowhead); 42 days post infection (DPI). DAPI (blue); Scale bar: 100 µm. (I) FACS sorting of PDGFRβ-positive

cells from cultures of the adult human cerebral cortex for subsequent live-imaging (see Figure 1G-G'). Left panel: FACS plot depicting the isotype controls for the analysis of PDGFRβ- and CD34-expression in a culture derived from adult human cerebral cortex. Right panel: FACS plot depicting PDGFRβ- and CD34-positive as well as negative fractions. CD34-negative/PDGFRβ-positive cells were sorted. (J-J') Induction of a neuronal phenotype following co-expression of Sox2 and Mash1 in cell cultures from the adult mouse cerebral cortex. Note that cells expressing Mash1 (red arrowheads) or Sox2 (green arrowheads) only are devoid of βIII-tubulin (white). Mash1 (Dsred), Sox2 (GFP). Scale bar: 100 µm. (K) Quantification of the effect on βIII-tubulin expression and morphology following DsRed, Sox2, Mash1 or combined Sox2 and Mash1 expression in cultures from adult mouse cerebral cortex. Cells were categorized for exhibiting a flat polygonal, round without processes, or neuronal morphology with processes. Histogram depicts the percentage of βIII-tubulin-positive among reporter-positive cells (n=4). Error bars are SEM. Control and Sox2-positive cells were analysed by counting >100 cells per experimental replicate, of which none were βIII-tubulin immunoreactive.

Figure S2

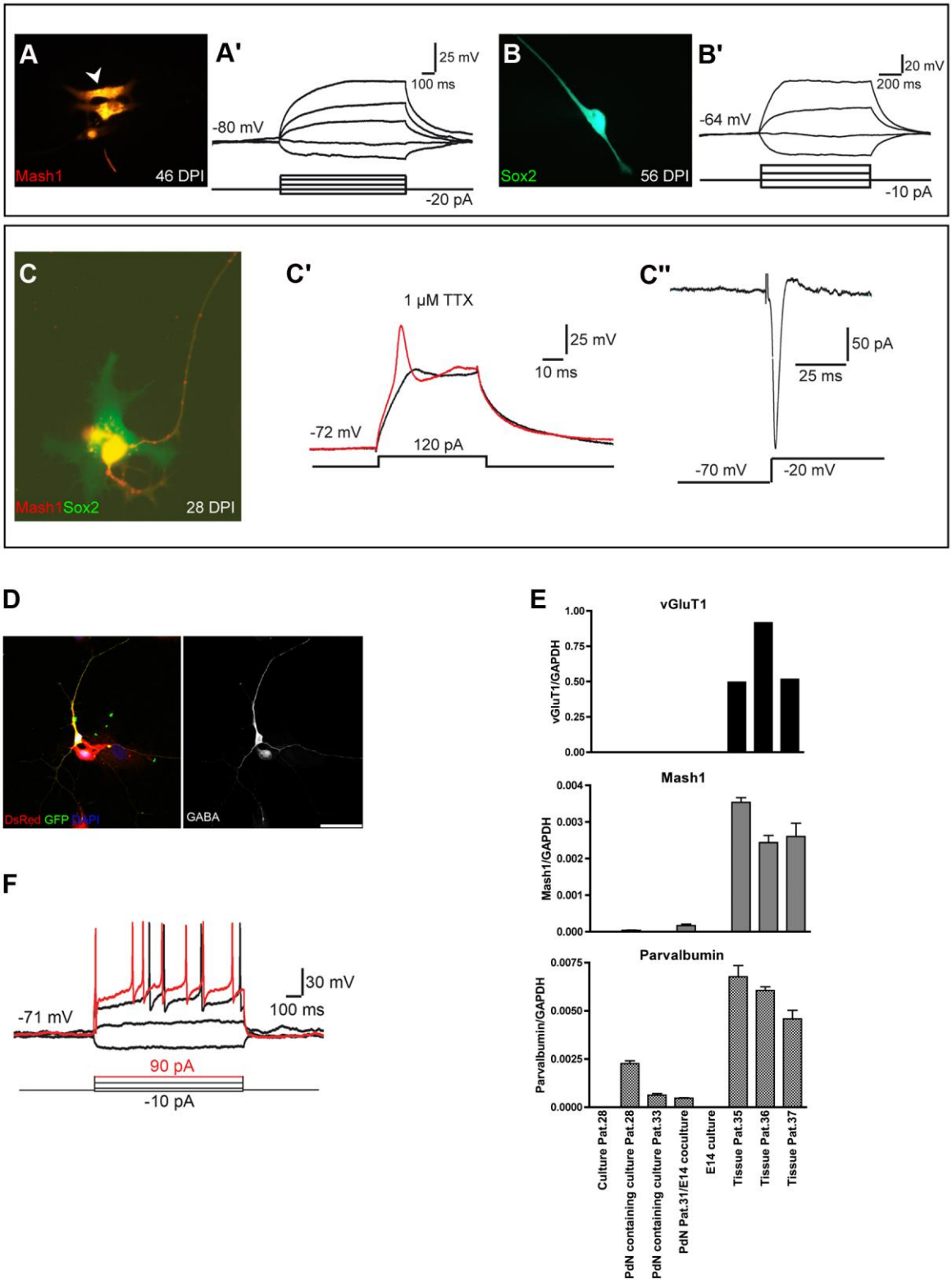

Figure S2. Functional properties of mouse and human PdiNs. (A) Example of a Mash1-only expressing human pericyte-derived cell. (A') Current-voltage relation of

the same cell (arrowhead), following step-depolarisation. Note the passive response of the cell. (B) Example of a Sox2-only expressing human pericyte-derived cell. (B') Passive current-voltage relation of the same cell. (C) Example of a hPdiN co-expressing Sox2 and Mash1 (yellow). (C') Following a step-current injection, the cell fires a single action potential (red trace) that is blocked by TTX (black trace). (C'') TTX-sensitive sodium currents recorded in voltage-clamp. For comparison of electrophysiological properties of hPdiNs following co-culture with mouse cortical neurons see Figure 2C. (D) GABA immunoreactivity in a hPdiN following Sox2 (green) and Mash1 (red) co-expression (left panel, DAPI blue) and GABA (white, right panel); 42 DPI. Scale bar: 50µm. (E) Quantitative RT-PCR analysis of the human glutamatergic neuron marker vGluT1 (vesicular glutamate transporter 1), human Mash1, and human parvalbumin in control pericyte culture, cultures containing PdiNs from 2 different patients, hPdiNs in co-culture with mouse E14 cortical neurons, mouse E14 cortical neurons alone, and tissue cortical samples from 3 patients. Note the extremely low expression levels of vGluT1, the low expression of Mash1, and the considerable expression level of the interneuron specific calcium binding protein parvalbumin in hPdiN containing cultures compared to cortical tissue. Expression was normalized to mRNA levels of GAPDH. (F) Example of action potential firing of a Sox2 and Mash1 co-transduced mouse PdiN in response to current injection in current-clamp.

Video S1. Direct observation of neuronal reprogramming of PDGFRβ- sorted pericyte-derived cells from the adult human brain by continuous live imaging in culture. Note the change in morphology of a cell co-expressing Sox2 and Mash1 (blue arrow) during reprogramming. Post-imaging immunocytochemistry for βIII-

tubulin (white) confirms the neuronal identity of the reprogrammed cell at the end of live imaging (see also Figure 1F).

Table S1. Summary of electrophysiological properties of Sox2 and Mash1 reprogrammed human and murine PdiNs.

	Human cells (no co-culture)	Human cells (co-culture)	Mouse cells
Resting potential V_R (mV)	-54 ± 13	-66 ± 11	-73 ± 10
Input resistance (MΩ)	2510 ± 919	2075 ± 940	811 ± 577
Number of cells exhibiting action potentials	12 /17	12 /12	6 /6
Number of action potentials / 1000 ms	1.3 ± 1.1	3.8 ± 6.8	5.0 ± 3.2
Action potential amplitude (mV)	56 ± 18	71 ± 30	77 ± 16
Peak Na^+ current (pA)	318 ± 268	912 ± 534	n.d .
Average age (days post infection)	35 ± 6	41 ± 5	32 ± 7

Supplemental Experimental Procedures

Surgery

Brain tissue was acquired directly from the operating room. Specimens of cerebral cortex (frontal and temporal lobe) of 30 patients aged between 19 and 70 years of both sexes were obtained from standard neurosurgical approaches to deep-seated, non-traumatic, non-malignant lesions or epilepsy surgical procedures. The study was approved by the ethical committee of the Medical Faculty of the LMU Munich and written informed consent was obtained from all patients.

Cell cultures from adult human and mouse cerebral cortex

After removal of the meninges, tissue was dissected and dissociated mechanically. Subsequently, cells were centrifuged for 5 min at 1000 rpm, re-suspended, and plated in a medium consisting of DMEM high glucose with GlutaMAX (Gibco), 20% fetal calf serum (Gibco), penicillin/streptomycin (Gibco). Originally, we also added 10 ng/ml epidermal growth factor (EGF, Roche) and fibroblast growth factor 2 (FGF2, Roche) to the medium to enhance the cell proliferation during expansion. However, we noted that pericyte-derived cells could be expanded without treatment with these factors with similar reprogramming efficiencies (data not shown) and thus both factors were omitted from the medium. After expansion for 2-3 weeks as adherent culture under normoxygenated conditions, cells were harvested using trypsin/EDTA (Gibco) and either plated onto poly-D-lysine (Sigma-Aldrich) coated glass coverslips at a density of 50,000 cells per coverslip (in 24-well plates; BD Biosciences) in the same medium as above for reprogramming experiments or passaged for further expansion (up to five times in this study). As for the human specimens, gray matter of the mouse cerebral cortex was dissociated mechanically. The subsequent steps of culturing were identical.

Coculture with E14 mouse cortical neurons

For co-culture experiments, E14 mouse cerebral cortices were dissected and dissociated mechanically with a fire-polished glass Pasteur pipette. Mouse cells were added to the human cultures 20 days after retroviral transduction at a density of 10.000-50.000 cells per coverslip. Electrophysiological recordings and/or immunocytochemistry were performed 39-47 days post retroviral infection. Experiments were repeated with 3 independent patients.

Retroviral transduction

Retroviral transduction of cultures was performed 2-3 h after plating on glass coverslips, using VSV-G (vesicular stomatitis virus glycoprotein)-pseudotyped retroviruses encoding neurogenic fate determinants as described previously (Heinrich et al., 2011). Mash1 or Sox2 were expressed under control of an internal chicken β-actin promoter with cytomegalovirus enhancer (pCAG) together with DsRed or GFP behind an internal ribosomal entry site (IRES). Expression of the transcription factors was confirmed by immunocytochemistry (data not shown). For control, cultures were transduced with a virus encoding only DsRed behind an IRES (pCAG-IRES-DsRed). For fate-mapping experiments a Sox2 construct without reporter was used (pLIB-Sox2), kindly provided by Dr. Wernig (Stanford, CA, USA). Twenty four hours after transduction, the medium was replaced by a differentiation medium consisting of DMEM high glucose with GlutaMAX, penicillin/streptomycin and B27 supplement (Gibco). Cells were allowed to differentiate under low oxygen conditions (5% O_2, 5% CO_2) (Galaxy 170R, New Brunswick).

Immunocytochemistry

Cell cultures were fixed in 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 15 min at room temperature. Cells were first pre-treated in 0.5% Triton X-100 in PBS for 30 min, followed by incubation in 10% goat serum and 0.5% Triton X-100 in PBS for 30 min. Primary antibodies were incubated on specimen for 1 h at room temperature or overnight at 4°C in 10% goat serum, 0.5% Triton X-100 in PBS. After extensive washing in PBS, cells were incubated with appropriate species- or subclass-specific secondary antibodies conjugated to fluorophores. Coverslips were finally mounted onto a glass slide with an anti-fading mounting medium (Aqua Poly/Mount; Polysciences, Warrington, PA).

Immunohistochemistry

Human brain tissue specimens were transferred into 4% paraformaldehyde and incubated for 48 h at 4° C. Thereafter, specimens were embedded in Tissue-tek (OCT compound, Sakura) and 20µm thick cryostat sections were cut. Mice were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA). Brains were collected, shortly post-fixed in 4% PFA and 20µm thick cryostat sections were cut. Sections were incubated with primary antibodies over night at 4ºC. After washing, secondary staining was performed using appropriate secondary antibodies. The following primary antibodies were used: chicken anti-Green Fluorescent Protein (Aves Labs, 1:1000), rabbit anti-Red Fluorescent Protein (Chemicon, 1:500), rabbit monoclonal anti-PDGFRβ (Cell Signalling, 1:200), mouse anti-Vimentin (Dako, 1:600), mouse anti-αSMA (Sigma-Aldrich, 1:400,), rabbit anti-NG2 (Millipore, 1:400), rabbit anti-hGFAP (Sigma-Aldrich, 1:600), rabbit anti-Sox2 (Chemicon, 1:1000), mouse anti-βIII-tubulin (Sigma-Aldrich, 1:400), mouse anti-Mash1 (Jackson-Price, 1:200), rabbit anti-CD146 (Abcam, 1:400), mouse anti-CD31 (Dako, 1:100), rat anti-CD31 (BD Pharmingen, 1:400), mouse anti-MAP2 (Millipore, 1:200), mouse anti-NeuN (Millipore, 1:200), rabbit anti-GABA (Sigma, 1:500), rabbit anti-Calretinin (Millipore,1:500), rabbit anti- vGluT1 (Synaptic Systems, 1:1000), and chicken anti-β-galactosidase (Abcam, 1:1000). Secondary antibodies conjugated to: Alexa Fluor 488 (Invitrogen, 1:500), Cy3, Cy5, FITC, TRITC (Jackson ImmunoResearch, 1:500), or biotin (1:500, Jackson ImmunoResearch or Vector Laboratories) for 1h in the dark at room temperature, followed by extensive washing in PBS. Following treatment with secondary antibodies conjugated to biotin, cells were subsequently incubated for 2h at room temperature with AMCA streptavidin (1:200, Vector Laboratories). Nuclei were stained with 4′,6-Diamidino-2-phenylindole (DAPI) (Sigma Aldrich, 1:10000).

Transgenic animals and tamoxifen induction

Postnatal Tg:TN-AP-CreERT2:R26R^{NZG} and Tg:TN-AP-CreERT2:R26R^{EYFP} mice (Dellavalle et al., 2011) were injected subcutaneously with 0.25mg of tamoxifen, diluted in corn oil for 3 consecutive days at 6, 7 and 8 days after birth to induce recombination.

Microscopy

Immunocytochemical and immunohistochemical stainings were first examined with an epifluorescence microscope (BX61, Olympus) equipped with the appropriate filter sets. Stainings were further analyzed with a LSM710 laser-scanning confocal microscope (Carl Zeiss,). Digital images were captured using the ZEN software (Carl Zeiss). Cell counts were performed using a 40X objective in at least five fields of view randomly selected from each coverslip. At least 3 independent experiments were counted.

Flow cytometry and FACS sorting

Human brain-derived cells were detached from the culture dish using 0.25% trypsin for 4-6 minutes and subsequently 1-3 x 10^5 cells were resuspended in 100 µl staining solution (PBS plus 0.5% BSA). For surface marker analysis, primary antibodies (PE-conjugated CD140b (PDGFR-β) (1:100, BD Biosciences) and APC-conjugated CD34 [1:100, BD Biosciences] were added individually and cells were incubated for 20 min at 4°C. After washing three times in staining solution, cells were resuspended in 500 µl staining solution and subjected to surface marker analysis using a FACS Aria (BD). The same setup was used to sort cells after co-staining with conjugated antibodies against CD34 and CD140b. APC-conjugated and PE-conjugated isotype control antibodies (1:100, AbD Serotec) were used to gate the proper populations.

CD34-negative/CD140b-positive as well as double negative cells (CD34-negative/CD140b-negative) were sorted. Following sorting, cells were plated on PDL-coated glass cover slips on 24-well plates.

qRT-PCR

Total RNA was extracted with RNeasy Plus MicroKit (Qiagen), according to the manufacturer's instructions. One μg of total RNA was retro-transcribed using Super-ScriptIII Reverse Transcriptase (Invitrogen) and random primers. Each cDNA was diluted one to ten, and 1μl was used for each real-time reaction. Messenger RNA quantitation was performed on a LightCycler480 (Roche) using the Light Cycler TaqMan Master kit (Roche) according to the manufacturer's instructions. The amount of each gene was analyzed in triplicate. Data analysis was performed with the ΔΔCt method (Livak and Schmittgen, 2001).

Electrophysiology

Perforated patch-clamp recordings were performed as described previously (Schinder et al., 2000). Cells were visualized with an epifluorescence microscope (Axioskop2, Carl Zeiss) and pictures of the recorded cells were acquired using a digital camera (AxioCam, Carl Zeiss).

Time-lapse video microscopy

Time-lapse video microscopy of sorted PDGFRβ-positive human cells was performed with a cell observer (Zeiss) at a constant temperature of 37^{o}C and 8% CO_2. Phase contrast images were acquired every 5 minutes, and fluorescence pictures every 7.5 hours for 14 days using a 10x phase contrast objective (Zeiss), and an AxioCamHRm camera with a self-written VBA module remote controlling Zeiss AxioVision 4.7

software (Rieger et al., 2009). Movies were assembled using Image J 1.42q (National Institute of Health, USA) software and are played at speed of 3 frames per second.

Statistics

Statistical analyses were performed by Student's two-tailed paired t-test using GraphPrism 4 software. For each analysis at least 3 independent experiments were performed.

Supplemental References

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Supplemental Movies and Spreadsheets

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