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Efficient induction of functional neurons from adult human fibroblasts

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Cellular reprogramming is a rapidly developing technology by which somatic cells are turned into pluripotent stem cells or other somatic cell types through expression of specific combinations of genes. This allows for the generation of patient-specific cell lines that can serve as tools for understanding disease pathogenesis, for drug screens and, potentially, for cell replacement therapies. Several cellular models of neurological disorders based on induced pluripotent cells (iPS cells) have been developed, and iPS-derived neurons are being explored as candidates for transplantation. Recent findings show that neurons can also be induced directly from embryonic and postnatal somatic cells by expression of defined combinations of genes. This conversion does not occur through a pluripotent stem cell stage, which eliminates the risk for tumor formation. Here, we demonstrate that functional neurons can be generated via direct conversion of fibroblasts also from adult individuals. Thus, this technology is an attractive alternative to iPS cells for generating patient- and disease-specific neurons suitable for disease modeling and autologous transplantation.

Induced pluripotent stem cells (iPS cells) obtained by reprogramming of adult human fibroblasts[1,2] are currently being used to develop models of a wide range of neurological diseases including spinal muscular atrophy, ALS, Parkinson disease and schizophrenia. Such cellular models will serve as valuable tools for understanding disease pathogenesis and progression.[3-5] The extent of genetic and epigenetic reprogramming and the mechanism for induction of pluripotency remains to be elucidated.[6-11] Nevertheless, the iPS technology has progressively been refined and human iPS cells can now be generated from different cell sources using methods that no longer rely on viral vectors or transgene integration.[12-16] These technical advancements have made iPS-derived neurons more suitable for replacement therapy, although they are still associated with substantial risk for tumor formation.[16-18]

Another approach for production of neurons from somatic cells is lineage reprogramming, in which one type of mature, differentiated somatic cell is directly transformed into another without passing through a pluripotent stage. Recent reports show that functional neurons, termed induced neurons, iNs, can be generated from mouse and human fetal and postnatal fibroblasts via direct conversion using defined sets of transcription factors such as Ascl1, Brn2 and Myt1l,[19-21] and subtype specific neurons can be obtained by varying the combination of transcription factors used.[20,22] Because of the ease of the technology, remarkably fast neural conversion and lack of a pluripotent stem cell intermediate, iN cells could become an attractive alternative to iPS cells. However, whether the iN technology can be used to produce functional neurons from fibroblasts obtained from adult humans using the same combination of factors is unknown.

Here we used fibroblasts from six healthy adults ranging from 23 to 65 y of age. The fibroblasts were converted into neurons using the same three factors, Ascl1, Brn2 and Myt1l that previously have been shown to induce functional neurons from embryonic and postnatal fibroblasts.[19-21] We demonstrate direct conversion of the adult human fibroblasts to functional iNs with comparable efficiency as when human embryonic and postnatal were used as starting material. Interestingly, we observed no decrease in conversion efficiency with age, as fibroblasts from the older individuals converting with efficiency comparable to fibroblasts from the younger individuals. Thus, iN cells have the potential to be useful both for disease modeling and in future applications of cell replacement therapy.

Results and Discussion

We first used cultures of distal derived lung fibroblasts originating from a healthy individual in the age range of 45–65 y (For details, see Materials and Methods and **Table 1**). This line, as well as the other adult human fibroblast lines subsequently used in this study, have previously been characterized and shown to express α-SMA, a protein involved in contractile apparatus and prolyl 4-hydroxylase, an enzyme involved in collagen synthesis, but not SM22, an actin-binding protein that is expressed at high

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Table 1. Clinical characterization

Name	Technique	Age (years of age)	Sex (M/F)	Distal fibroblasts
Ind1	Transplantation biopsy	45–65	F	yes
Ind2	Transplantation biospy	45–65	F	yes
Ind3	Transbronchial biopsy	29	M	yes
Ind4	Transbronchial biopsy	23	F	yes
Ind5	Transbronchial biopsy	23	M	yes
Ind6	Transbronchial biopsy	34	F	yes

levels in smooth muscle cells.[23] Before being used in the present set of experiments, the homogeneous fibroblast properties of the cells when cultured in fibroblast (MEF) medium, as well as when cultured in neural induction (N2B27) medium, were confirmed by staining for Collagen 1 and Collagen 3 (**Fig. 1A**). The absence of contaminating neural progenitors and neural crest cells in the fibroblast cultures was confirmed by qPCR as previously described in reference 20 (**Fig. 1B**). The cells were then used for conversion by delivering lentiviral vectors coding for Ascl1, Brn2 and Myt1l. In cultures transduced with conversion factors and subsequently grown in neural media, cells with elongated, neuron-like morphology became visible already 3–4 d after transgene activation, similar to what we observed for embryonic fibroblasts.[20] The cultures were fixed and analyzed by immunocytochemistry 12 and 20 d after transduction. Cells with characteristic neuronal morphology and expressing the neuronal markers βIII-tubulin and MAP2 were detected at both timepoints (**Fig. 1C**). In parallel control cultures, containing fibroblasts from the same individual and passage but not infected with conversion factors (otherwise treated identically), MAP2- and βIII-tubulin-expressing neurons were never observed (**Fig. 1C**).

We next performed similar conversion experiments on distal lung fibroblasts from a total of six healthy individuals of ages ranging from 23 to 65 y old (**Table 1**).[23] Based on cell number at each passage, theoretically a minimum of 70 x 10^6 to 142 x 10^6 fibroblasts from each individual were available at passage 5–6, which we used for conversion. We found that the fibroblasts from all six individuals converted into neurons expressing MAP2 and βIII-tubulin (**Fig. 2A**), with efficiencies similar to what has been reported for embryonic and postnatal fibroblasts (**Fig. 2D**).[7,8] We observed no difference in conversion efficiency between transbronchial and transplantation biopsies or gender. Interestingly, there was no correlation between age and conversion efficiency, as fibroblasts from the older individuals converting with efficiency comparable to fibroblasts from the younger individuals (**Fig. 2D**). Thus, iNs can be generated also from somatic cells of adult and elderly individuals.

To determine whether the neurons produced from the human adult-derived fibroblasts were functional, we performed whole-cell patch-clamp recordings on iN cells from three of the six individuals (Ind 1, 3 and 6) between 23 and 34 d after transduction. The induced neurons derived from all three individuals exhibited electrophysiological properties of functional neurons. Depolarizing current injection induced action potentials in ~47% of recorded cells (n = 60 cells in total, **Fig. 2B**) and in voltage-clamp mode, step depolarization gave rise to fast inactivating inward and outward currents characteristic of sodium and delayed rectifier potassium currents, respectively (**Fig. 2B**). Biocytin labeling confirmed the neuronal morphology of the recorded cells (**Fig. 2C**).

Taken together, our results demonstrate that functional neurons can be generated by direct conversion of fibroblasts from adult humans using combined expression of Ascl1, Brn2 and Myt1l. The derivation of iNs from patients with various neurological disorders should provide a novel platform for disease modeling. Moreover, patient-specific iNs could potentially be suitable for autologous cell replacement therapies. Because the iNs do not pass via a proliferative pluripotent stem cell stage, which has the advantage of circumventing the tumorigenicity concerns associated with grafting of pluripotent cells such as iPS cells,[18] the number of neurons that can be obtained is finite. However, we successfully used fibroblasts 5–6 passages after being isolated from each individual, which should provide a sufficient number of fibroblasts for potential therapeutic applications. If iN cells in the future can be obtained without the use of lentiviral vectors or transgene integration, they could provide a safe source of clinically applicable, patient-specific neurons in regenerative therapies for neurological disorders.

Materials and Methods

Subjects and sampling of tissue. Distal derived lung fibroblasts from 6 healthy individuals with no clinical history of lung disease were used. All sampling was done under approval of local Ethics committee (Dnr 413/2008 and 412/03). In four of these subjects (Ind 3–6), transbronchial specimens were taken with biopsy forceps (Olympus FB211D) guided by a fluoroscope. Biopsies were chopped into small pieces and immediately transferred to cell culture medium [DMEM supplemented with 10% FBS, Gentamicin and Amphotericin B (Gibco BRL)]. The tissue pieces were allowed to adhere to the plastic of cell culture flasks for 4 h, and then kept in cell culture medium at 37°C until outgrowth of fibroblasts was observed. For the remaining two individuals (Ind 1–2), fibroblasts were isolated from explants. Alveolar parenchymal specimens were collected 2–3 cm from the pleura in the lower lobes, i.e., from the same location where transbronchial biopsies were taken. Vessels and small airways were removed from the peripheral lung tissues and remaining tissues were chopped into small pieces. After rinsing, parenchymal pieces were allowed to adhere to the plastic of cell culture flasks for 4 h and were then kept in cell culture medium in 37°C cell incubators until outgrowth of fibroblasts was observed. For the first 2 passages, the cells were always split 1:3 (T25). Cells were then transferred to T75 and passaged 1:2 for subsequent passages. Passage 5–6 fibroblasts were used for experiments.

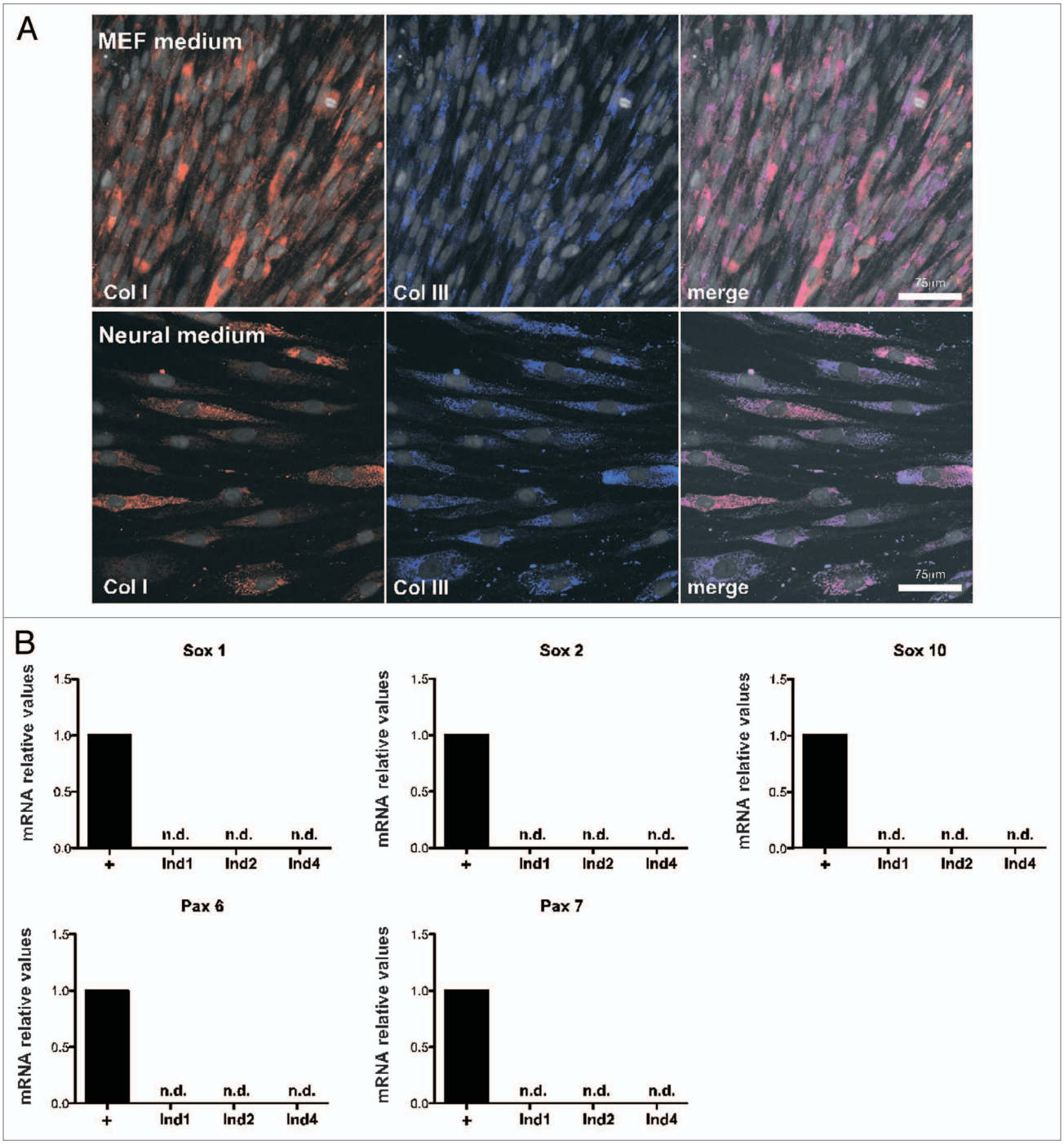

Figure 1A–B (For part C, see page 3314). Direct conversion of adult human fibroblasts into human induced neural (iN) cells. (A) Fibroblast identity was validated by expression of collagen type I and collagen type III (ColI, ColIII). Fibroblasts were analyzed both when cultured in mouse embryonic fibroblast (MEF) medium and when cultured in neural induction (N2B27) medium. White indicates DAPI nuclear stain. (B) qRT-PCR analysis showed absence of the neural progenitor and neural crest markers *SOX1*, *SOX2*, *SOX10, PAX6* and *PAX7* from non-converted fibroblasts (cultured in N2B27 for 20 d; normalized to positive control (+), non passaged primary hEF cultures for *SOX* genes and whole head of human embryo for P*AX* genes).

Viral vectors and neural conversion. Doxycycline-regulated lentiviral vectors expressing mouse cDNAs for AsclI, Brn2 and Myt1l have been described in reference 20. The doxycycline regulated system includes a separate lentiviral vector expressing a TET-ON transactivator (FUW.rtTA-SM2, Addgene) that was always co-transduced in the conversion experiments. The titers of the vectors used in this study were in the range of 1.3 x 10^8–2.9 x 10^9 TU/ml. A MOI of 5 for ABM and 10 for Fuw was used for conversion.

For neuronal conversion, fibroblasts were plated in MEF medium at a density of 10,000 cells/cm^2 in tissue culture plates (NUNC) coated with 0.1% gelatine. Neuronal conversion was

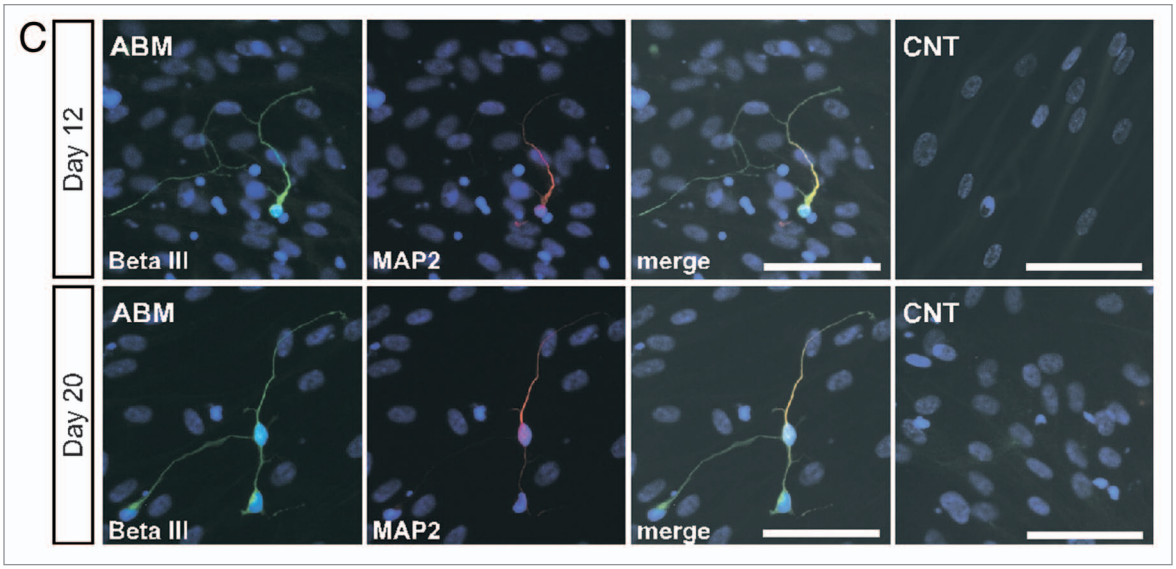

Figure 1C (For parts A–B, see previous page). Direct conversion of adult human fibroblasts into human induced neural (iN) cells. (C) Neurons expressing βIII-tubulin (green) and MAP2 (red) obtained by direct conversion of adult fibroblasts at day 12 and day 20 after Ascl1, Brn2 and Myt1l expression was activated. In parallel cultures treated the same but without delivering transgenes no neurons were ever observed (right part C). Blue indicates DAPI nuclear stain. Scale bars = 75 μm.

performed as previously described in reference 20, using N2/B27 (Stem Cell Sciences) as neural induction media.

Immunocytochemistry. Cells were fixed in 4% paraformaldehyde (PFA) and pre-incubated for 30 min-1 h in blocking solution (5% normal serum and 0.25% triton-X in 0.1 M KPBS). The primary antibodies (MAP2 1:500, βIII-tubulin 1:5,000) were diluted in the blocking solution and applied over night in 4°C. Fluorophore-conjugated secondary antibodies (Molecular Probes or Jackson Laboratories) were diluted in blocking solution and applied for 2 h followed by 3 rinses in KPBS. All images were obtained using a Leica inverted microscope.

Electrophysiology. Recordings were performed as previously described in reference 20. Briefly, converted cells grown on coverslips were constantly perfused with heated (32–34°C), gassed (95% O_2, 5% CO_2) bath solution (pH 7.2–7.4, 295–300 mOsm) containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4 and 25 glucose. Recording pipettes were filled with solution (pH 7.2–7.4, 295–300 mOsm) containing (in mM): 122.5 potassium gluconate, 12.5 KCl, 10.0 KOH-Hepes, 0.2 KOH-EGTA, 2 MgATP, 0.3 Na_3-GTP and 8 NaCl, resulting in pipette resistances of 3–5 MΩ. Voltage-gated sodium channels were blocked using 1 μm tetrodotoxin (TTX, Tocris).

Quantitative RT-PCR. Total RNA was isolated using the RNeasy Micro kit (Qiagen) according to the supplier's recommendations. For each sample, 300 ng of RNA was used for reverse transcription performed with random primers and SuperScriptIII (Invitrogen). SYBR green quantitative real-time PCR was performed with LightCycler 480 SYBR Green I Master (Roche) in a 2-step cycling protocol. Data were quantified using the ΔΔCt-method and averaged upon normalization to GAPDH and β-actin expression. The specificity was confirmed by analyzing the dissociation curve and by validation in human embryonic tissue. Primer sequences were the same as previously reported in reference 20.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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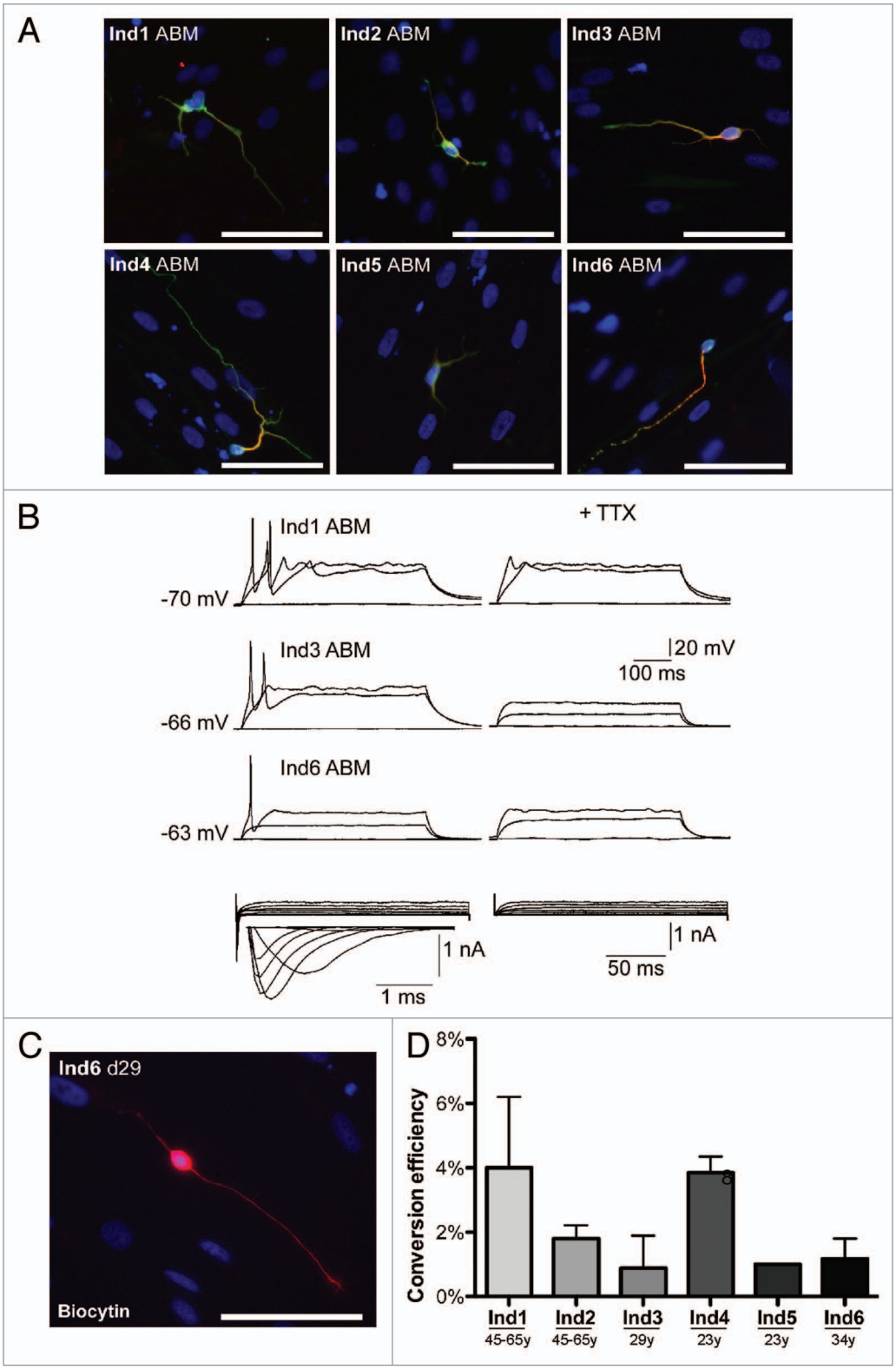

Figure 2. Efficiency and functionality of direct conversion from six individual adults. (A) iNs expressing ßIII-tubulin (green) and MAP2 (red) were formed by direct conversion of fibroblasts (Passage 5–6) from all six individuals, Ind1–6 (n = 4–6 for each individual). Blue indicates DAPI nuclear stain. (B) Representative traces of action potentials induced by step injection of depolarizing current (10 pA increments) and of whole cell currents induced by depolarizing voltage steps from -60 mV to +20 mV in 10 mV increments. Action potentials and Na^+ currents were blocked by bath application of TTX (right part). (C) Biocytin filled hiN cell confirmed neuronal morphology of recorded cells. (D) Conversion efficiency estimation of hiN formation at days 12, Passage 5–6 (n = 4 for Ind1; n = 2 for Ind2; n = 2 for Ind3; n = 2 for Ind4; n = 1 for Ind5; n = 3 Ind6). Scale bars: (A) 75 μm; (B) 50 μm.

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