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Neuronal differentiation of human iPS-cells in a rat cortical primary culture

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We tested the neuronal differentiation of human iPS-cells under *in vitro* conditions. For this purpose we pre-differentiated human (h) iPS-cells into neural stem cells and co-cultivated them with a cortical primary culture from embryonic rats. After 2 days of co-cultivation a certain number of hiPS-cells exhibited a clear neuronal morphology combined with expression of betaIII-tubulin and doublecortin. In addition, we found hiPS-cells without neuronal differentiation and cells already expressing betaIII-tubulin but not having yet distinctive axonal and dendritic processes. Human neuronal progenitors, starting neuronal differentiation, were contacted by both neuronal processes from rat neurons and oligodendrocytes, indicating a possible instructive influence by the primary culture on human cells. After 7 days of co-cultivation, however, we observed a complete degeneration of human iPS-derived cells and phagocytosis by microglial cells. Immunocytochemical stainings surprisingly revealed that microglial cells of the cortical primary culture express both CD8 and T-cell receptors.

Key words: human iPS-cells, cortical primary culture, neuronal development, MTC02, betaIII-tubulin, DCX, microglia, CD8, TCR

INTRODUCTION

Induced pluripotent stem cells (iPS-cells) are in the focus of stem cell research since their discovery by Takahashi and Yamanaka (2006). Fibroblasts are transformed into stem cells by the overexpression of transcription factors like Oct4, Sox2, Klf4 and c-myc and the induced stem cells display features and self-renewal similar to ES-cells. This comprehends the *in vitro* induction of different cells representing the progeny of all three germ layers, the formation of teratoma in immunodeficient mice, and the integration in blastocysts and generation of chimeric animals (Zhou and Ding 2010, Sidhu 2011). In the last years the necessary conditions for induction of pluripotency have been defined more precisely and new protocols have been developed (O'Malley et al. 2009).

These protocols include e.g. the use of non-integrative adenovirus (Zhou and Freed 2009), of episomal vectors (Stadtfeld et al. 2008, Yu et al. 2009), recombinant proteins (Zhou et al. 2009; Kim et al. 2009) or mRNA (Warren et al. 2010). For the generation of tissue committed cells iPS- alike ES-cells are usually pre-differentiated into multipotent stem cells, which then in turn are further differentiated into specialized endo-, meso-, and ectodermal cells. One application area of iPS cells is the generation of autologous neural stem cells with the long-term perspective to use them for transplantation approaches in neurodegenerative diseases. Thus, first experimental studies have been performed, e.g. in Parkinson models (Fricker-Gates and Gates 2010, Hargus et al. 2010, Chang et al. 2011, Lindvall and Björklund 2011) and models for spinal cord injury in rats (Salewski et al. 2010, Tsuji et al. 2010).

These experimental models are suitable in order to evaluate the overall survivability of delivered cells and to assess potential positive effects on the facilitation of regenerative processes. More specific questions, however, as the velocity of neuronal differentiation, the expression of certain marker proteins or the interactions with neurons and oligodendrocytes at cellular

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level can be more easily determined under *in vitro* conditions as it was done for other stem cells (Janowski et al. 2011).

In this study we investigated therefore the neuronal differentiation of human iPS cells within a cortical primary culture. We used human iPS cells to generate neural stem cells and co-cultivated them with a cortical primary culture from rat embryos. Our results reveal, that NSC derived from human iPS cells have the capacity to differentiate into neurons very quickly in a rat brain environment. The emerged phenotype of human neurons was identical to that of cortical neurons from rats. However, a longer co-cultivation caused the complete degeneration of human iPS cells. The main characteristic of this degeneration is phagocytosis by microglia. The consequences on commonly used animal models for transplantation will be discussed.

METHODS

Generation of human iPS cells and neuronal pre-differentiation

The I-M5 hiPS cell line has been derived from MRC5 human fetal lung fibroblasts (ATCC, USA; 200 000 cells) transduced with the lentivirus-derived pSin-EF2-Oct4-Pur, pSin-EF2-Sox2-Pur, pSin-EF2-Nanog-Pur and pSin-EF2-Lin28-Pur13 plasmids (Addgene; Cambridge, USA) according to Yu and colleagues (2007). Viruses were produced by Vectalys (Labège, France; MOI between 7 and 23). The culture medium (Dulbecco's modified Eagle medium containing 10% fetal bovine serum) was modified to serum-free hESC medium containing 20 ng/mL bFGF (R&D Systems) over one week. Transduced fibroblasts were seeded onto mouse embryonic feeder cells and grown in hESC medium. Colonies with ESC morphology appeared after 3 to 6 weeks and were clonally expanded for 5 weeks with mechanical passage every 5 to 7 days. Five colonies from MRC5 cultures were selected on a morphological basis and validated for the expression of hESC self-renewal genes and surface markers, and for pluripotency through embryoid bodies and teratoma formation in immunodeprived mice, before differentiation into the neural lineage. A full description of quality control can be found in Polentes and coauthors (2012). Neural stem cells (NSC) were derived using the dual SMAD inhibitor protocol described by Chambers and others (2009). Early neural precursors (ENPs) were produced in low-attachment cultures in the presence of Noggin (R&D Systems), the transforming growth factor-β pathway inhibitor SB431542 (Tocris Biosci.), bFGF (10 µg/mL, Preprotech) and hBDNF (20 µg/mL, R&DSystems). Then, neural progenitor cells (NPCs) were produced from ENPs in the presence of 20 ng/mL hBDNF for 7days.

Co-culture with a cortical primary culture

Brains from embryonic rats (E16–E18) were removed from the skull and both cortices were dissected from the brain stem and transferred into DMEM medium with 10% FBS (both Biochrom). Cortical tissue was mechanically minced and centrifuged (320 g for 2 min). Tissue pellets were resuspended in Hanks –buffer free of Ca^{2+} and Mg^{2+} and centrifuged again. After resuspension 1ml EDTA/PBS solution (Biochrom) was added before new centrifugation. Trypsination was performed by adding 2 ml Trypsin/EDTA solution (0.25%/0.02%, Biochrom) to the 6 ml culture suspension and 2 min incubation at room temperature under gentle agitation. After resuspension in DMEM with 10% FBS samples were squirted twice through a syringe equipped with a 23 gauge needle. After resuspension in the same medium and counting with a Neubauer hemocytometer, one million cells were seeded per well of a 12 well plate (1 ml/well) containing Matrigel (Becton Dickinson) coated glass cover slips. Cells were grown under standard conditions at 37°C and 5% CO_2. After 1 day *in vitro* (DIV) the DMEM medium was changed to 800 µl StartV medium (Biochrom). Immediately afterwards human iPS cells (10^5 I-M5 cells in 100 µl StartV medium) were added. Medium was changed every 2 days with 800 µl StartV medium. The cortical primary culture was fixed after 2 or 7 days of co-culturing with 4% paraformaldehyde for 15 min at room temperature.

Immunocytochemistry

After fixation, potential IgG binding sites were saturated with 10% donkey serum (Sigma). Cells were incubated with primary antibodies in PBS with 1%

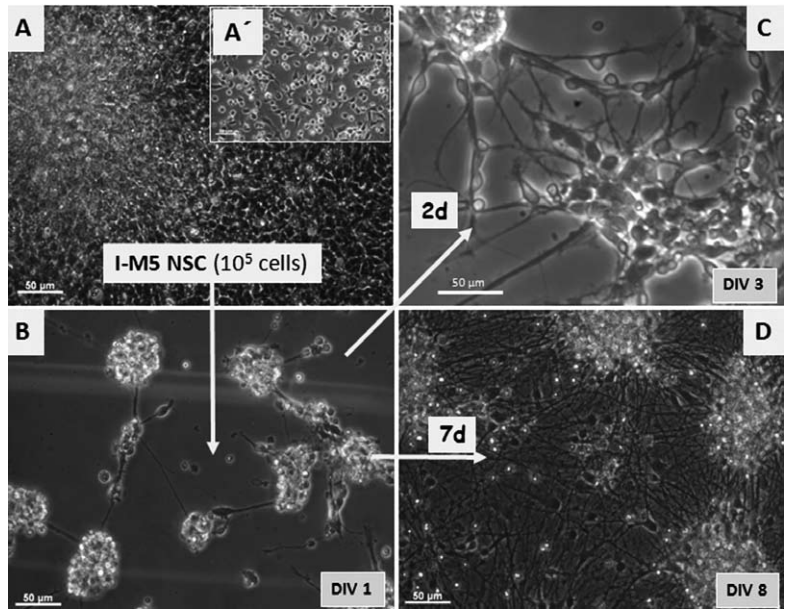

Fig.1. Co-culture of neural stem cells from human iPS cells with cortical primary cells from the embryonic rat brain. Neural stem cells from I-M5 hiPS cells (10^5 I-M5 cells) were co-cultivated with primary cortical cells for 2 or 7 days. (A) Neural stem cells at high density and shortly after passaging (A´). (B)−(D) Cortical primary cells after 1 DIV (B) and with co-cultured hiPS derived NSC 3 days (C) and 8 days (D) *in vitro* (DIV). Scale bar is 50 µm.

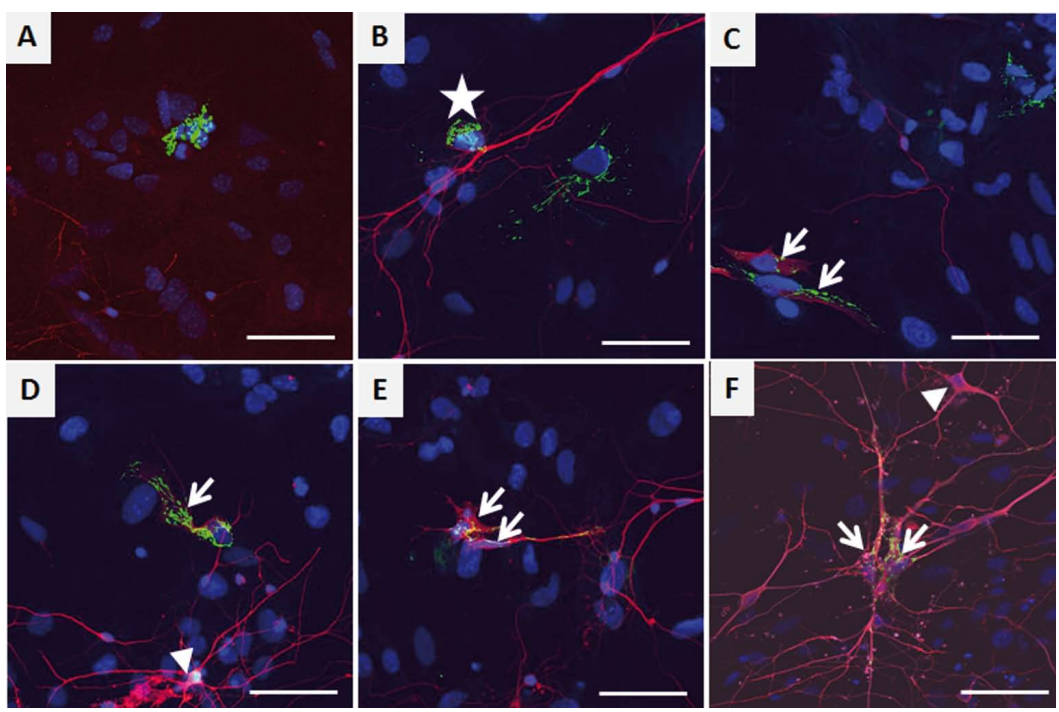

Fig. 2. Neuronal differentiation of hiPS-NSC after 2 days in co-culture with cortical primary cells from the embryonic rat brain. Human cells derived from iPS cells were labeled by a specific antibody against mitochondria (MTC02, green). After 2 days of co-culture human cells displayed two different phenotypes. Cells not expressing βIII-tubulin (A, B) and cells expressing detectable levels of βIII-tubulin but still different in their shape from cortical neurons (red, arrows in C, D). Undifferentiated human cells were often found in close contact with processes of rat cortical neurons (asterisk in B). Other hiPS-NSC in the same cultures had already acquired a fully mature neuronal phenotype, with high expression of βIII-tubulin (arrows in E, F) alike primary cortical neurons (arrowheads in D, F). Nuclei stained with DAPI. Scale bar is 50 µm.

donkey serum at room temperature overnight. The following antibodies were used: MTC02 (mouse; 1:200 Dianova); DCX (goat; 1:300 Santa Cruz); βIII Tubulin (mouse or rabbit; 1:1 000 Promega); NG2 (rabbit; 1:300 Chemicon); Iba1 (goat; 1:500 Abcam) IB4-Alexa568 (1:1 000 Mobitec); Ox42 (mouse; 1:200 Serotec); CD8 (mouse; 1:1 000 Serotec); TCR (mouse; 1:500 Serotec)

After washing, primary antibodies were revealed by binding with secondary antibodies (donkey; 1:500, Dianova), labeled with Cy2, Cy3 or Cy5 for 2 h at room temperature. Nuclei were stained by DAPI (1:15 000; Mobitec) for 10 min. All used antibody had shown specificity in our laboratory in multiple experiments with known antigens. Non-specific binding of secondary antibodies was controlled by staining cells and brain tissues without primary antibodies. Finally, glass cover slips were dehydrated by increasing ethanol concentrations (70–100%), briefly submerged in xylol and mounted in DPX (Fluka). Cortical primary cultures were analysed with a confocal Laser Scanning Microscope (LSM 5 Pascal Exciter with associated software Version 4.0; Zeiss, Germany) equipped with helium/neon lasers emitting at at 543 nm/633 nm, respectively, an argon laser emitting at 488 nm and a diode emitting 405–425 nm. Multitracking was used to avoid cross-talk between channels.

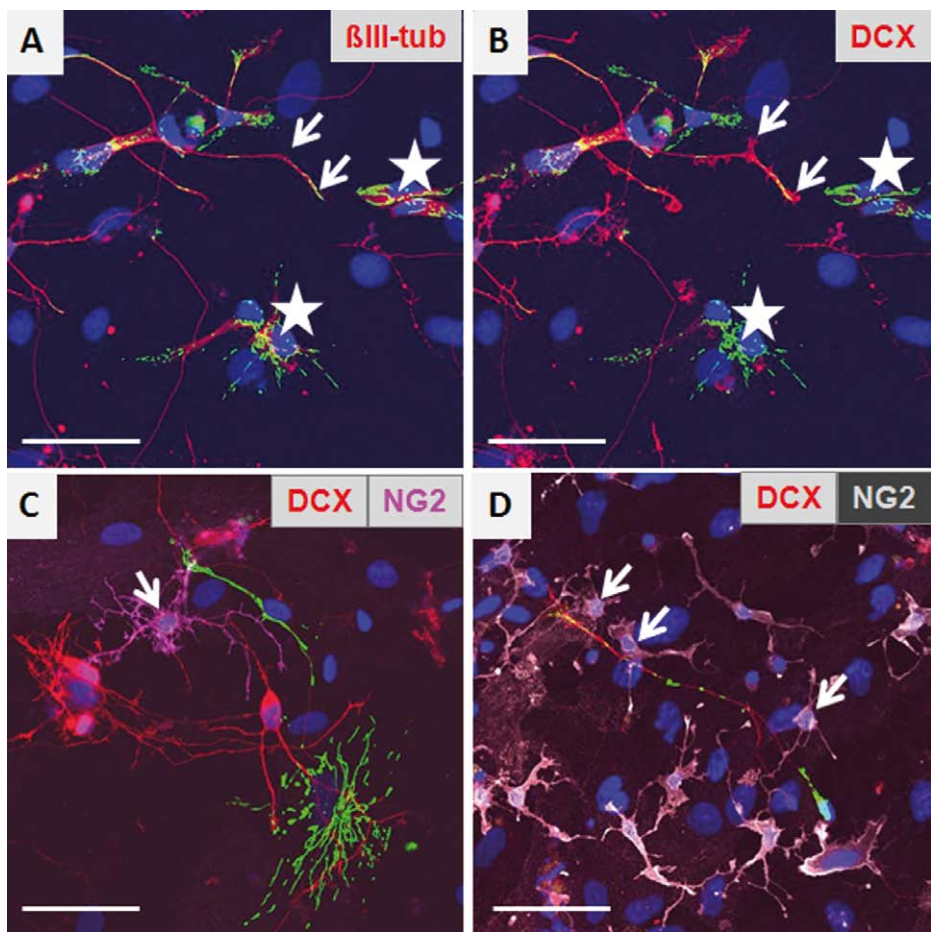

Fig. 3. Contact by oligodendrocyte progenitors and double-expression of βIII-tubulin and DCX after 2 days of co-culture. As typical for young neurons, human neurons (MTCO2, green) expressed both, βIII-tubulin (red; A) and DCX (red; B). Quantification revealed an overall number of 22.6% hiPS derived cells double-positive for βIII-tubulin and DCX. βIII-tubulin expression increases faster than DCX (asterisks in A, B). In contrast to βIII-tubulin, DCX is also expressed in dendritic and axonal arborisations (arrows in A, B). (C) and (D) illustrate the contact of rat-derived oligodendrocyte progenitors to neurons. (C) rat oligodendrocyte progenitors (NG2, arrow) contacting rat primary neurons (DCX). (D) Several oligodendrocyte progenitors (NG2, arrows) contact the DCX positive developing axon of a hiPS-derived neuron. NG2 staining of oligodendrocyte progenitors is displayed in grey tone in (D). Nuclei stained with DAPI. Scale bar is 50 µm.

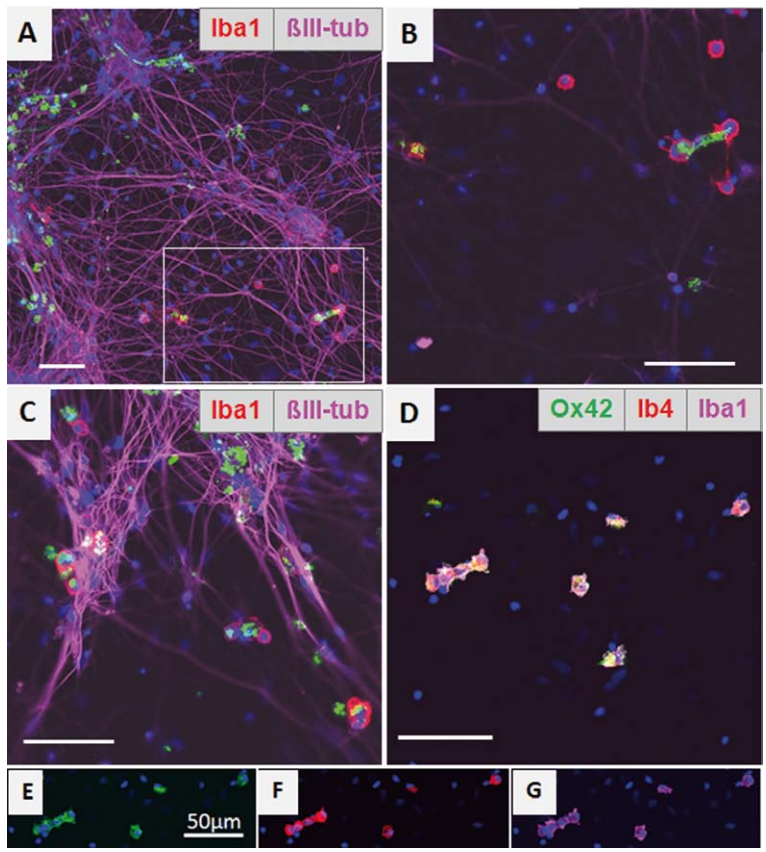

Fig. 4. Phagocytosis of human cells by rat microglial cells. (A)–(C) After 7 days of co-culture with rat brain cells, hiPS-derived cells (MTCO2, green) degenerated and were subjected to phagocytosis by rat microglia cells. Rat microglia is stained by Iba1 (red in A–C, magenta in D, G). The neuronal network of rat cells is revealed by βIII-tubulin (magenta). (B) is a higher magnification of insert in (A). (E)–(G) Single channels of the merged picture in D confirming co-labeling with Ox42 (E) and Ib4 (F) of Iba1 (G) positive microglia. Nuclei stained with DAPI. Scale bars is 50 µm.

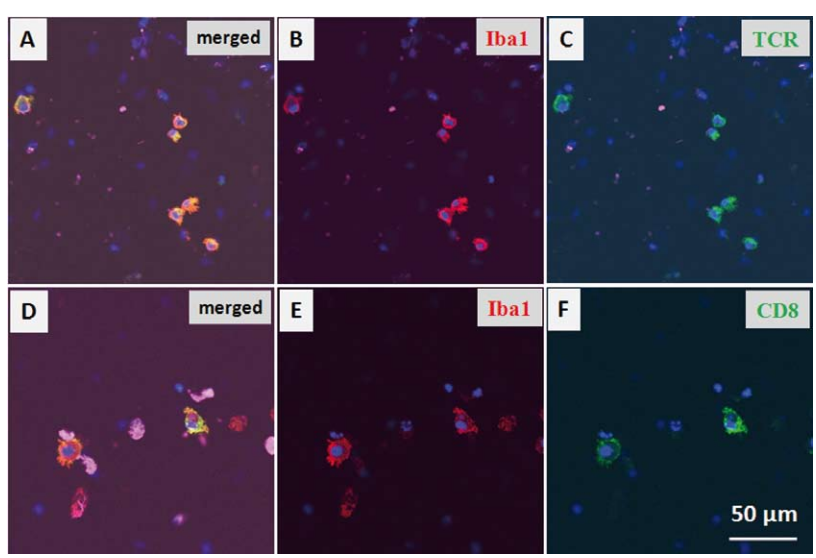

Fig. 5. Rat microglial cells express T-cell markers. Microglial cells (Iba1) from the cortical primary culture express the T-cell receptor (TCR; A–C) and CD8 (D–F). Both T-cell markers are expressed on microglia independently of the presence of human cells in the cortical primary culture. Pictures are taken from confocal z-stack planes where βIII-tubulin positive neurons (magenta) are not visible. Scale bar is 50 µm.

RESULTS

Preparation of a rat cortical primary culture

Culture of neural cells from the cortex of embryonic rats on Matrigel-coated cover slips results in a three-dimensional cell layer consisting of young neurons, astrocytes, oligodendrocytes and microglia. Neural stem cells and neuronal precursor cells isolated from the developing cortex aggregate to form neurospheres, which give rise to the development of a three-dimensional network of cells (Fig. 1). Neurons maturing in this network express βIII-tubulin and DCX and built up tight connections with surrounding astrocytes and oligodendrocytes (for further details please refer to Braun et al. 2006).

Co-culture of hiPS-NSC with the cortical primary culture

Pre-differentiated human iPS-NSC (10^5 I-M5 cells) were seeded on DIV 1 and co-cultivated with the cortical primary culture for 2 days (short culture) or 7 days (long culture). At this time hiPS-NSC cells had integrated into the neuronal network, without being discriminable under living conditions by phase contrast microscopy (Fig. 1C). Immunocytochemical staining with a specific antibody against human mitochondria (MTC02) enabled the detection of human cells at different stages of neuronal development (Fig. 2). We detected compact human cells without any expression of βIII-tubulin and displaying only short or no processes (Fig. 2A, B). Interestingly, these immature cells were contacted by processes from cortical neurons (Fig. 2B). Two days old co-cultures also contained human cells positive for βIII-tubulin that had started neuronal differentiation. In contrast to βIII-tubulin negative cells, these human cells already exhibited an expanded cell shape. However, their phenotype is still clearly different from that of cortical neurons in the primary culture (Fig. 2C, D). Finally, we detected iPS cells with phenotype of mature neurons. These human neurons were not distinguishable from cortical neurons on morphological criteria and showed a high expression of βIII-tubulin characteristic for neurons (Fig. 2E, F).

Quantification of 304 human iPS derived cells in confocal images revealed that 163 cells (53.6%) were undifferentiated, 72 cells (23.7%) were developing neurons already expressing βIII tubulin and 69 cells (22.6%) were differentiated neurons with a cell shape not distinguishable from rat cortical neurons and expressing both βIII tubulin and DCX.

The neuronal differentiation of human cells correlated with increased expression of βIII-tubulin and DCX, whereby expression levels of βIII-tubulin increased faster than those of DCX (asterisk in Fig. 3A, B). DCX, however, seems to be more essential for the emergence of tiny arborisations of axonal and dendritic processes (arrows in Fig. 3A, B). These processes were contacted by NG2-positive oligodendrocytes of the cortical primary culture. Figure 3C shows a contact between an oligodendrocyte and an axonal process of a cortical neuron. Figure 3D demonstrates how an axon of a human iPS- derived cell is connected with several oligodendrocyte progenitors expressing NG2.

Phagocytosis of hiPS-derived cells by rat microglia positive for CD8 and TCR

Since human NSC underwent a very fast differentiation within 2 days of co-culture with cortical primary cells, the integration of human cells into the neuronal network was followed over 7 days. Surprisingly, no living hiPS-derived neurons were detectable. Instead, human cells were collapsed and subjected to phagocytosis by Iba1 positive microglial cells from the cortical primary culture (Fig. 4A–C). Co-labeling of Iba1-positive cells by Ox42 (CD11b/c) and the isolectin IB4 confirmed that Iba1-positive cells were rat microglia cells (Fig. 4D–G). In order to exclude a possible presence of T-cells in the cortical primary culture, we performed immunocytochemical stainings against the T-cell markers CD8 and T-cell receptor (TCR). Surprisingly, Iba1 positive microglial cells co-expressed CD8 or TCR (Fig. 5).

DISCUSSION

We have recently investigated the fate of human iPS derived neural stem cells after transplantation into rats after experimental stroke (Polentes et al. 2012). Cells were proofed before transplantation by quantitative PCR-analysis, embryoid body formation, karyotype analysis and teratoma formation for their identity of pluripotent stem cells. Grafts developed neurons within four weeks, received afferents from the host and

sent axons to homotopic brain regions. Here, in parallel to that transplantation study in rats we have investigated the same hiPS-derived NSC under *in vitro* conditions. For that purpose hiPS cells generated from MRC5 fibroblasts were differentiated into NSC using dual inhibition of SMAD pathways. Human NSC were then co-cultivated with a cortical primary culture from embryonic rats. After 2 days, 22.6% of NSC had already differentiated into cells with a neuronal phenotype and high expression levels of βIII-tubulin and DCX. After longer co-cultivation, however, human cells were engulfed by microglia from the cortical primary culture.

Cortical primary cultures represent an *in vitro* model, that contains all neural cell-types including neurons, astrocytes and oligodendrocytes, in addition to microglia. Under our culture conditions, nestin positive neural precursors, together with young neurons aggregate into neurospheres and generate a neuronal network consisting of mature neurons, astrocytes and young oligodendrocytes (Braun et al. 2006). Human iPS-derived cells were added when formation of the neuronal network was just starting. These in vitro conditions considerably differ from the *in vivo* conditions of an adult brain, mainly by lack of blood-derived molecules and cells. However, after acute brain lesions, such as stroke or trauma, conditions may arise that promote pronounced regeneration (Jablonska and Lukomska 2011). This regeneration is accompanied by migration of neural stem cells and microglia as well as an activation of astrocytes (Kokaia et al. 2006, Thored et al. 2009, Xue et al. 2009, Lindvall and Kokaia 2011). The developing cortical primary culture reflects, at least partly, this cell migration and differentiation of neural cells under *in vitro* conditions.

The neuronal development of human cells came along with distinct transformations of their cell morphology. Initially compact NSC rapidly started to shape broad processes and up-regulated the expression of βIII-tubulin. Then, human cells developed long βIII-tubulin-positive processes and were no longer distinguishable from cortical neurons by morphological criteria.

One crucial criterion for neuronal differentiation is the expression of the cytoskeleton marker DCX. While βIII-tubulin is expressed at low levels also in non-neuronal cells, e.g. mesenchymal cells (personal observation), the expression of DCX seems to be restricted to young neurons (Brown et al. 2003, Couillard-Despres et al. 2005). In fact, we found a conspicuous up-regulation of both markers along with the differentiation of hiPS derived NSC into neurons. This correlates with the development of young neurons within the cortical primary culture and with neuronal development of neural stem cells located in the lateral ventricle wall (Bédard and Parent 2004, Yang et al. 2004, Braun et al. 2006). DCX, as also underlined by our results, is involved in the formation of axonal and dendritic spines (Jin et al. 2010).

Interestingly, tight contacts were formed between hiPS cells and axonal processes from cortical neurons before human cells had started to up-regulate βIII-tubulin. These contacts may facilitate the release of neurotrophic factors that have the potential to accelerate neuronal differentiation (Memberg and Hall 1995). The emergence of dendritic and axonal processes by human cells preceded the contact by oligodendrocyte precursor cells (OPC) derived from the primary culture. We refer to these contacts as a necessary milestone for the functional integration of hiPS-derived neurons into the neuronal network formed by rat cells. NG2 cells mainly represent oligodendrocyte progenitor cells (OPC). These cells give rise to mature oligodendrocytes but OPC may also retain as NG2 positive cells representing a lifelong ability to proliferation combined with an increased resistance to insults and toxicity (Dawson et al. 2003, Dzwonek 2005). Also, there is an accumulating number of indications, that NG2 cells represent a more heterogeneous population of precursor cells, which might give rise also to neurons and astrocytes depending on cues coming from the surrounding tissue (Sypecka et al. 2009, Ju et al. 2012). Furthermore, NG2 cells are able to interact with neurons by receiving signals through glutamatergic and GABAergic synapses (Mangin and Gallo 2011). Finally, microglia cells have been reported to express NG2 in addition to Ox42 after focal injection of LPS into rat brain (Zhu et al. 2012). The morphology of the rat NG2 cells in our system, however, favors the development into oligodendrocytes.

It becomes obvious, that *in vivo* investigations of human cells in rodents cannot discover the full developmental potential of the delivered cells, since this xenotransplantation triggers a strong immune reaction from the host against transplanted cells (Bühnemann et al. 2006). The co-cultivation of

human cells with cortical primary cells from rats creates conditions that mimic those of a xenotransplantation, however in the absence of host circulating cells. This might explain why longer co-culture leads to the degeneration of human iPS-derived cells and phagocytosis by rat microglia. Whether this "rejection process" is initiated exclusively by microglia or chemokines secreted by other cells, e.g. astrocytes are also involved, remains an open question.

It is commonly accepted that rejection is initiated and driven by T-cells (Ingulli 2010). We did not find any signs for the presence of T-cells in the rat cortical primary culture. However, to our surprise many microglia cells were positive for CD8 or TCR. To our knowledge, the expression of TCR on microglia is not described.

In contrast, CD8 positive microglia has already been found in different *in vivo* paradigms. CD8 positive macrophages / microglia were first described after focal ischemia in rat brain (Jander et al. 1998, Schroeter et al. 2001). The same group described the recruitment of CD8+ macrophages during Wallerian degeneration after crush injury to the sciatic nerve (Jander et al. 2001). These CD8+ macrophages co-expressed ED1 and CD11b, the latter being the target of the Ox42 antibody we used in our study for microglia identification. Furthermore, infiltration of CD8 positive microglia / macrophages was described after traumatic brain injury in rats (Zhang et al. 2006). Finally, the recruitment of CD8-positive macrophages was observed after chronical experimental autoimmune encephalomyelitis in rats (Schroeter et al. 2003, Hiraki et al. 2009). Microglia has also been described to locally differentiate into antigen presenting dendritic cells in the brain (Fischer and Reichmann 2001) and the downregulation of microglia activity by minocycline leads to an extended survival of transplanted porcine cells in the rat brain (Michel-Monigadon et al. 2010). All these *in vivo* data from the literature were attained in rat models. This makes it very likely, that our *in vitro* data about phagocytosis of human cells by rat microglia have relevance for the rejection of transplanted human cells in the rodent brain. Since microglia cells as descendants of the mesodermal germ layer are present in the developing cortex, they are also present in the cortical primary culture from the very beginning. It remains open whether special MHC signals trigger the phagocytosis of human iPS derived cells by rat microglia or whether cell death of human cells is necessary for the activation of microglia.

CONCLUSIONS

Our results demonstrate that hiPS cell-derived neuronal precursors are able to differentiate into neurons very fast when seeded on a primary culture of rat cortex. Although we have indications that this differentiation occurs after transplantation into rats more slowly (Polentes et al. 2012), cortical primary cultures reveal the great potential of human iPS-cells for neuronal differentiation.

In vitro studies have the potential to reveal certain aspects of cell differentiation or cell-cell contacts more effectively than *in vivo* studies mainly due to greater sensitive levels. However, the great differences between fetal cells as used in this study and the adult brain have to keep in mind.

Further, it becomes obvious, that the full potential of human iPS-cells can be evaluated only under autologous transplantation conditions. Only under these circumstances one can expect minimal host versus graft reaction. Thus, ironically one of the greatest advantages of human iPS-cells, namely the autologous origin for the recipient, cannot be tested under preclinical *in vivo* conditions, since immunological reactions of the mice or rat limit the long-term development of grafted human cells. One direction to circumvent greater immunological problems in experimental studies is the creation of iPS-cells from rat or mice and testing them under autologous conditions (Welstead et al. 2008, Okita et al. 2010). Our results show that microglial cells may play an important, so far unrecognized, role in host *versus* graft reactions under xenogaft conditions. Besides this, cortical primary cultures may serve as a feasible *in vitro* model not only for revealing such immunological reactions but also other cell-cell interactions between the neural tissue and exogenous human iPS-cells.

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