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SHORT REPORT

Polysialic acid modification of the synaptic cell adhesion molecule SynCAM 1 in human embryonic stem cell-derived oligodendrocyte precursor cells

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Abstract
Oligodendrocyte precursor cells (OPCs) are the progenitors of myelinating oligodendrocytes in brain development and repair. Successful myelination depends on the control of adhesiveness during OPC migration and axon contact formation. The decoration of cell surface proteins with the glycan polysialic acid (polySia) is a key regulatory element of OPC interactions during development and under pathological conditions. By far the major protein carrier of polySia is the neural cell adhesion molecule NCAM, but recently, polysialylation of the synaptic cell adhesion molecule SynCAM 1 has been detected in the developing mouse brain. In mice, polySia-SynCAM 1 is associated with cells expressing NG2, a marker of a heterogeneous precursor cell population, which is the primary source for oligodendrocytes in development and myelin repair but can also give rise to astrocytes and possibly neurons. It is not yet clear if polySia-SynCAM 1 is expressed by OPCs and its occurrence in humans is elusive. By generating uniform human embryonic stem cell-derived OPC cultures, we demonstrate that polySia is present on human OPCs but down-regulated during differentiation into myelin basic protein-positive oligodendrocytes. PolySia on NCAM resides on the isoforms NCAM-180 and NCAM-140, and SynCAM 1 is identified as a novel polySia acceptor in human OPCs.
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Introduction

Developmental myelination and myelin repair after demyelination are mediated by oligodendrocyte precursor cells (OPCs). OPCs are characterized by markers such as PDGFRα and the chondroitin sulfate proteoglycan NG2 (Dubois-Dalcq et al., 2008; Franklin and Ffrench-Constant, 2008; Nishiyama et al., 2009; Trotter et al., 2010). Differentiation protocols for the

Abbreviations: Endo, endosialidase; MBP, myelin basic protein; NCAM, neural cell adhesion molecule; OPC, oligodendrocyte precursor cells; PDGFRα, platelet-derived growth factor receptor alpha; polySia, polysialic acid; polyST, polysialyltransferase; SynCAM, synaptic cell adhesion molecule.

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production of human embryonic stem cell-derived (hESCd) OPCs have been described and successful myelination was achieved by grafting of hESCd-OPCs into brain or spinal cord of myelin-deficient shiverer mice (Nistor et al., 2005; Hu et al., 2009a,b). Remyelination and improved functional recovery after transplantation of hESCd-OPCs have also been demonstrated in rat spinal cord injury (Keirstead et al., 2005; Sharp et al., 2010) and recently, myelination by human induced pluripotent stem cell (iPSC)-derived OPCs has been demonstrated in the mouse model (Wang et al., 2013; Douvaras et al., 2014). Thus, human stem cell-derived OPCs are on the way to be used in replacement therapies for treating demyelinating diseases (Goldman et al., 2012; Fox et al., 2014). Besides environmental and axonal cues, the efficiency of myelination is determined by the capacity of OPCs to migrate and to interact with non-myelinated axons.

A regulatory factor of particularly these OPC properties is the modification of the cell surface with the unique glycan polysialic acid (polySia or PSA). The major carrier of polySia is the neural cell adhesion molecule NCAM and its main function is the modulation of cell surface interactions during brain development and plasticity (Rutishauser, 2008; Schnaar et al., 2014). PolySia on OPCs supports migration towards demyelinated lesions (Wang et al., 1994; Nait-Oumesmar et al., 1999, 2007; Zhang et al., 2004). Accordingly, enhanced polysialylation improved the migration capacity of mouse ESC- and human iPSC-derived OPCs (Glaser et al., 2007; Czepiel et al., 2014). In the course of oligodendrocyte maturation and myelin formation, polySia on OPCs is down-regulated and myelination is impairedwhen the down-regulation of polySia is prevented (Fewou et al., 2007). Moreover, polySia on axons inhibits myelination *in vitro* (Charles et al., 2000) and chronically demyelinated axons in multiple sclerosis lesions re-express polySia, whereas shadow plaques with partial remyelination are polySia-negative (Charles et al., 2002). Thus, polySia on OPCs facilitates OPC recruitment and the presence of polySia on OPCs and on axons may act as a negative regulator of myelin formation.

PolySia synthesis is mediated by two independently regulated polysialyltransferases (polySTs), ST8SIA2 and ST8SIA4. Both enzymes show high acceptor specificity and therefore polySia is restricted to only few carrier molecules (Mühlenhoff et al., 2013). In addition to the major polySia carrier NCAM, we recently described the ST8SIA2-mediated polysialylation of a fraction of the synaptic cell adhesion molecule SynCAM 1 within a subset of NG2 cells of the early postnatal mouse brain (Galuska et al., 2010; Rollenhagen et al., 2012). NG2 cells are able to communicate with neurons via specialized neuron-NG2 cell synapses (Bergles et al., 2000; Karadottir et al., 2005; Kukley et al., 2007; Etxeberria et al., 2010). This axo-glial signaling may be involved in the onset of myelination (De Biase et al., 2010; Kukley et al., 2010). As SynCAM 1 is critical for assembly, organization and maintenance of neuronal synapses (Biederer et al., 2002; Stagi et al., 2010; Fogel et al., 2011) it may also contribute to the formation of synapses between neurons and NG2 cells. And because polySia attenuates adhesive interactions, it has been proposed that polysialylation of SynCAM 1 modulates the assembly or disassembly of neuron-NG2 cell synapses (Galuska et al., 2010). However, many aspects of OPC biology differ fundamentally between mice and humans (Sim et al., 2009) and so far, the polySia modification of SynCAM 1 has only been shown in mice (Galuska et al., 2010; Rollenhagen et al., 2012). Therefore, the present study was designed to assess polySia acceptors in human OPCs generated from ESCs.

Materials and methods

hESC culture and differentiation to OCPs and oligodendrocytes

Human embryonic stem cells ES03 (hESCs, ES Cell International, National Stem Cell Bank Wisconsin) were maintained as described previously (Konze et al., 2014). The protocol for OPC differentiation was adapted from Hu et al. (2009a,b) (for details, see Supplementary material, extended methods).

Immunocytochemistry and microscopy

For immunocytochemistry hESCd-OPCs were cultivated in 24-well plates on glass coverslips coated with poly-L-ornithine/laminin (Sigma-Aldrich). Fixed cells were permeabilized with 0.1% Triton X-100, stained and imaged as described (Schiff et al., 2009). For details on antibodies, specificity controls and image acquisition, see Supplementary material, extended methods.

RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA from hESCd-OPCs was extracted using NucleoSpin RNA II kit (Macherey-Nagel) and reverse transcribed as described (Rollenhagen et al., 2012). Quantitative real-time PCR was performed as described previously (Konze et al., 2014). Relative mRNA levels of target genes were determined by normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward: 5′-ATGACATCAAGAAGGTGGTG-3′, reverse: 5′-CATACCAGGAAATGAGCTTG-3′). Amplification of ST8SIA2 and ST8SIA4 cDNA was performed using primers specific for ST8SIA2 (forward: 5′-TGACGCCCACAGCTTCG-3′, reverse: 5′-CATCCCGGGCATACTCCTG-3′) and ST8SIA4 (forward: 5′-GACAAAAGAAATAGCAAGAACTGAGGA-3′, reverse: 5′-CCGACTCAAAGACAATTCACC-3′), generating products of 62 bp and 76 bp, respectively. PCR products were separated on 6% non-denaturating polyacrylamide gels and visualized by ethidium bromide.

Immunoprecipitation, SDS-PAGE and Western blotting

hESCd-OPCs were lysed in 500 μl of 50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 5 mM EDTA, 2% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 200 U/ml aprotinin, 10 μg/ml leupetin and 2 mM phenylmethylsulfonylfluoride. After centrifugation cell lysates were mixed with 100 μl of mAb 735-conjugated tosylactivated magnetic dynabeads (Invitrogen), incubated shaking for 2 h at 4 °C, washed and eluted with Laemmli buffer. Immunoprecipitation (IP) of SynCAM or depletion of NCAM from cell lysates was performed accordingly, using Protein G Sepharose 4 Fast

Flow (GE Healthcare) and 10 μg of anti-SynCAM pAb or anti-NCAM mAb. For enzymatic removal of polySia, eluted proteins were treated with 6 μg/ml endosialidase (Endo, Stummeyer et al., 2005) for 30 min at 37 °C. SDS-PAGE (8%) and Western blotting were carried out as described (Galuska et al., 2010). For details, see Supplementary material, extended methods.

Results and discussion

hESCd-OPCs are positive for polySia, NCAM and SynCAM 1

The differentiation procedure started from hESCs cultured on γ-irradiated human fibroblasts as described previously

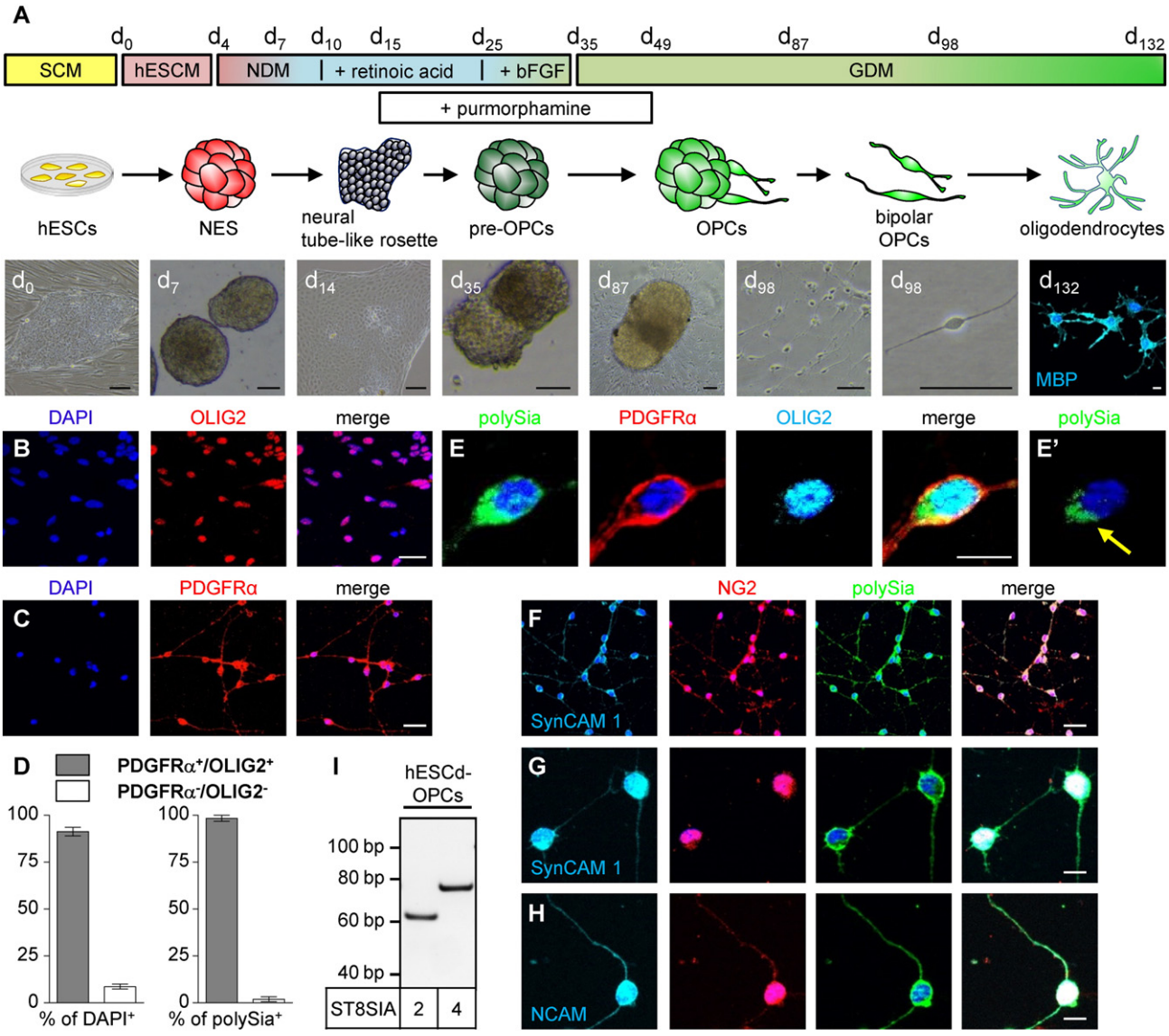

Figure 1 Efficient generation of polySia-positive OPCs from hESCs. (A) Schematic procedure for the directed differentiation of hESCs into OPCs and mature oligodendrocytes. Stem cell medium (SCM), hESC medium (hESCM), neural differentiation medium (NDM), glial differentiation medium (GDM), neuroepithelial spheres (NES). For details see Supplementary material, extended methods. Micrographs show representative images of cells at the indicated days of differentiation (d). d_0–d_{98}, bright field images, d_{132}, immunofluorescence staining of myelin basic protein (MBP). (B, C) Expression of the OPC markers OLIG2 and PDGFRα at d_{98}. Nuclear counterstain with DAPI (blue). (D) Evaluation of PDGFRα and OLIG2 double-positive or double-negative cells in hESCd-OPC cultures at d_{98} expressed as % of all DAPI- or polySia-positive cells, as indicated (mean ± SEM from n = 3 independent cultures). (E) Co-staining of polySia with OLIG2 and PDGFRα at d_{98}. (E′) Micrograph acquired with short exposure time highlights strong perinuclear polySia immunoreactivity (arrow) of the cell depicted in (E). (F–H) Staining for NCAM and SynCAM 1 in NG2- and polySia-positive cells. Scale bars, 50 μM (A) or 20 μM (B, C, E–H). (I) Bands obtained by reverse transcription PCR performed from d_{98} hESCd-OPC cultures with primers specific for *ST8SIA2* and *ST8SIA4*.

(Konze et al., 2014) and is depicted in Fig. 1A. After 98 days of differentiation (d_{98}) this procedure reproducibly generated cultures containing >90% hESC-derived bi- and multipolar cells positive for OLIG2, PDGFRα and NG2 (Fig. 1B–F). Although none of these markers on its own is exclusive to the oligodendrocyte lineage (Nishiyama et al., 2009; Trotter et al., 2010), the combined expression of these markers provides substantial evidence that the differentiation procedure gives rise to OPCs. To corroborate the largely uniform composition, d_{98} OPC cultures were screened for the presence of astrocytic or neuronally committed cells using GFAP-, Pax6-, ß-III-tubulin- and doublecortin (DCX)-specific antibodies. In contrast to neurally induced hESC-derived cultures with numerous Pax6-, ß-III-tubulin- and DCX-positive neural precursor cells (NPCs; see Supplementary material, Fig. S1), none of these markers could be detected in d_{98} OPC cultures. Virtually all cells identified as OPCs showed polySia immunoreactivity, while the few PDGFRα- and OLIG2-negative cells were essentially devoid of polySia signals (Fig. 1D–F). This is consistent with previous reports showing polySia on murine OPCs and human subventricular zone-derived glial progenitors *in vivo* (Nait-Oumesmar et al., 1995, 1999, 2007; Picard-Riera et al., 2002) and with a recent study demonstrating the presence of polySia in human iPSC-derived OPCs (Czepiel et al., 2014). PolySia was distributed over the whole cell body, including protrusions (Fig. 1E–H), but micrographs acquired with short exposure time indicated a particularly strong perinuclear signal (Fig. 1E′, arrow). In addition to NCAM, which so far is the only known polySia acceptor in the human brain (Cox et al., 2009), all hESCd-OPCs expressed SynCAM 1 together with NG2 and polySia (Fig. 1F–H). Analysis by RT-PCR indicated mRNA expression of the two mammalian polySTs ST8SIA2 and ST8SIA4 (Fig. 1I). PolySia on NCAM can be produced by both enzymes, whereas murine SynCAM 1 is polysialylated exclusively by ST8SIA2 (Rollenhagen et al., 2012). Therefore, the presence of both polyST transcripts indicates that hESCd-OPCs have the capacity to polysialylate NCAM and SynCAM 1.

PolySia in hESCd-OPCs is attached to NCAM and SynCAM 1

To identify polySia acceptors in hESCd-OPCs, total polysialylated proteins were isolated by immunoprecipitation (IP) with the polySia-specific mAb 735. Immunoblotting demonstrated broad signals characteristic for polysialylated proteins, ranging from ~95 to 220 kDa (Fig. 2A) (Mühlenhoff et al., 1996; Galuska et al., 2010; Rollenhagen et al., 2012, 2013). These signals were completely abolished by incubation of samples with Endo, which degrades polySia with high specificity (Stummeyer et al., 2005). Western blot analysis with NCAM-specific antibodies demonstrated that the strong polySia signal above ~140 kDa represents polySia-NCAM. After Endo treatment the polySia-NCAM smear resolved into two sharp bands corresponding to NCAM-140 and NCAM-180. This is the first evidence that human OPCs carry polySia on the same NCAM isoforms that are known to be polysialylated during postnatal development in mouse brain and human cortex (Oltmann-Norden et al., 2008; Cox et al., 2009).

NCAM depletion from hESCd-OPC lysates resulted in a clear reduction of the polySia signal above ~140 kDa, whereas the polySia signal centered around 110 kDa was not affected (Fig. 2B, left). Immunodetection with SynCAM-specific antibody on the same membrane resulted in bands corresponding to the lower polySia signal, which broadened towards lower apparent molecular masses upon polySia removal by Endo treatment (Fig. 2B, right). Reversely, IP with SynCAM-specific antibody resulted in polySia and SynCAM signals centered around 110 kDa (Fig. 2C). In addition, non-polysialylated SynCAM was detected in the lower molecular weight range between ~60 and 75 kDa. These signals correspond to the extensively glycosylated, but not polysialylated SynCAM

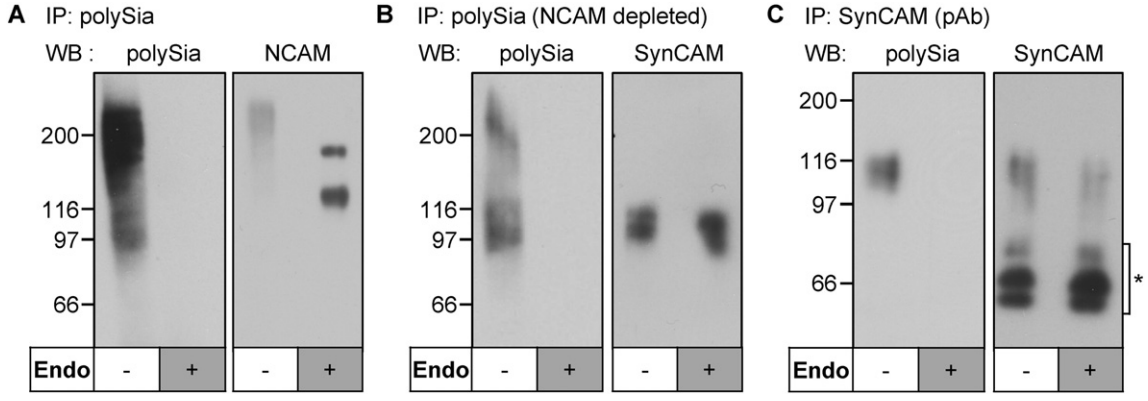

Figure 2 PolySia is attached to NCAM and SynCAM 1 in hESCd-OPCs. (A) IP of total polysialylated proteins from hESCd-OPC lysates with mAb 735-conjugated beads. Prior to SDS-PAGE, one part of each sample was treated with endosialidase (Endo +). Western blot developed with polySia-specific mAb 735, and, after stripping, with NCAM-specific mAb 123C3, as indicated. Due to steric hindrance in the presence of polySia, the binding of mAb 123C3 to its epitope in the extracellular domain of polysialylated NCAM is strongly reduced (Hildebrandt et al., 1998). (B) IP of polysialylated proteins from hESCd-OPC lysates after depletion of NCAM by IP with mAb 123C3. Western blot with mAb 735, and, after stripping, with SynCAM-specific pAb. Endo treatment as indicated [see (A)]. (C) IP from hESCd-OPC lysates with SynCAM-specific pAb. WB and Endo treatment as in (B). Asterisk marks polySia-negative SynCAM species. Molecular masses (kDa) of standard proteins are indicated.

species detected in rat and mouse brain samples (Biederer et al., 2002; Rollenhagen et al., 2012). The concordant polySia and SynCAM signals obtained by Western blot as well as the minor shift caused by the specific removal of polySia are in perfect agreement with the features described for polySia-SynCAM 1 isolated from mouse brain (Galuska et al.,

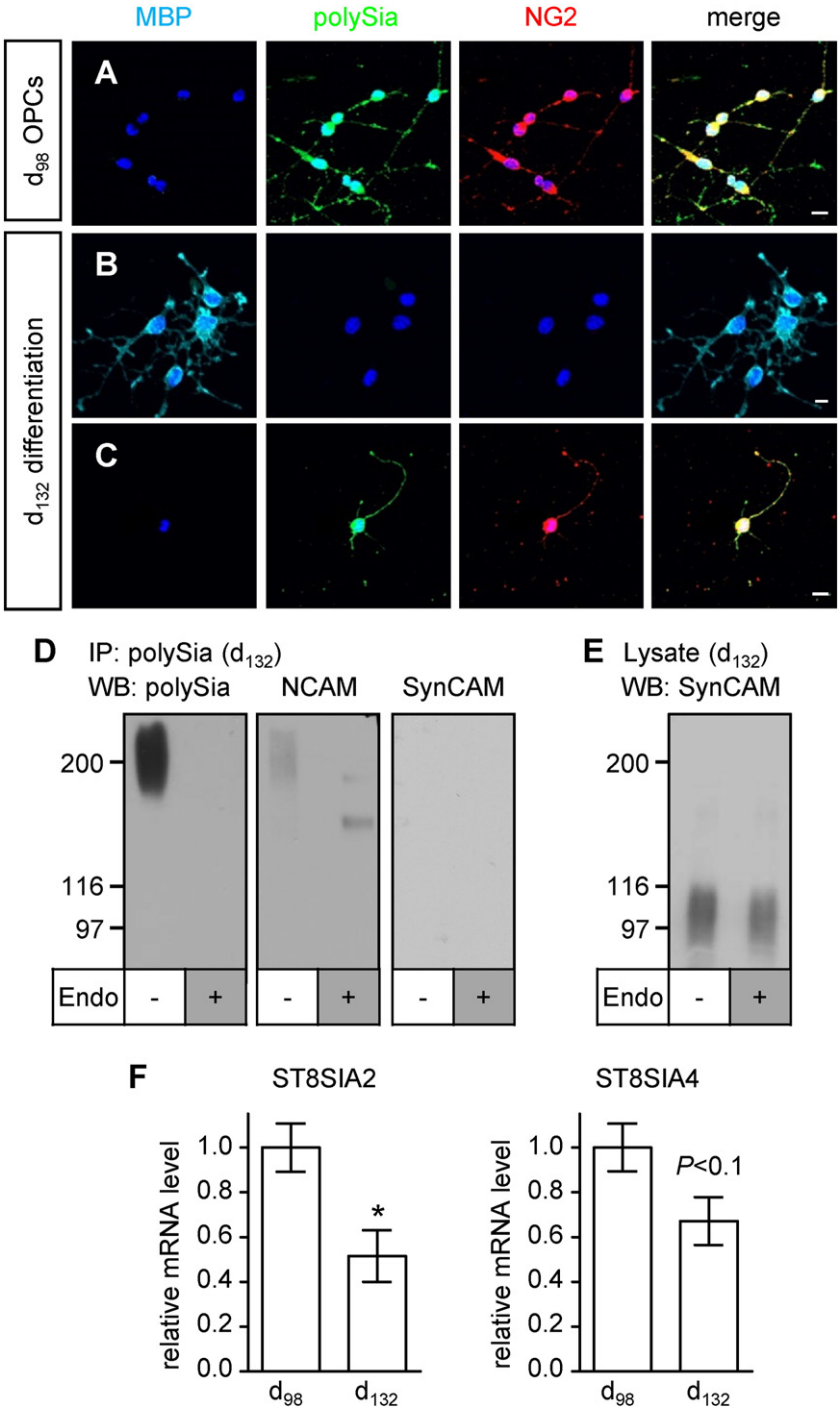

Figure 3 Polysialylation of NCAM and SynCAM 1 is down-regulated during differentiation of OPCs into mature oligodendrocytes. (A–C) Cells stained for myelin basic protein (MBP), NG2 and polySia in hESCd-cultures at d_{98} and d_{132} as indicated. Scale bars, 20 μM. (D) PolySia IP of d_{132} cultures and Western blot analysis of untreated or Endo-treated samples with polySia-, NCAM-, and SynCAM-specific antibodies. (E) Western blot analysis of SynCAM in the range of ~110 kDa in untreated or Endo-treated lysates of d_{132} hESCd cultures. Molecular masses (kDa) of standard proteins are indicated. (F) Relative mRNA level for *ST8SIA2* and *ST8SIA4* in hESCd-cultures at d_{98} and d_{132}. Values represent mean ± SEM from 3 independent replicates. *, significant differences with $P < 0.05$, *t*-test.

2010; Rollenhagen et al., 2012). Together, these data prove that human ESC-derived OPCs express polysialylated SynCAM 1 and provide the first evidence that SynCAM 1 is an acceptor for posttranslational modification with polySia in humans.

PolySia is down-regulated during oligodendrocyte maturation

PolySia expression of rodent OPCs is down-regulated during differentiation into mature myelinating oligodendrocytes (Trotter et al., 1989; Bartsch et al., 1990; Nait-Oumesmar et al., 1995; Wang et al., 1994). We therefore analyzed changes of polySia expression between d_{98} hESCd-OPCs and cultures differentiated until d_{132}. At d_{98}, almost all of the cells expressed OPC markers and had only few processes (see Fig. 1). In addition, none of the cells were immunopositive for MBP, which was assessed by mAb SMI-99, a marker of mature oligodendrocytes in rodents and humans (Sternberger et al., 1978; Back et al., 2001) (Fig. 3A). At d_{132} most of the cells had the appearance of mature oligodendrocytes with multiple branched processes and were positive for MBP, but not NG2 or polySia (Fig. 3B). Only some cells (~5%) remained positive for NG2 and polySia (Fig. 3C). In addition, a few scarce GFAP-immunoreactive cells (<1% of all $DAPI^+$ cells), but no neuronal, Pax6-, ß-III-tubulin- or DCX-positive cells were detected (see Supplementary material, Fig. S1). Despite the relatively high proportion of cells that acquired MBP expression in d_{132} cultures, cellular heterogeneity is an inherent confounding feature of pluripotent stem cell differentiation systems. As broadly discussed by, e.g., Solozobova et al. (2012) it is hardly possible to maintain cell type identity by *in vitro* differentiation approaches, like the one employed in the current study. Thus, for biomedical applications possible deviations from the oligodendrocytic specification achieved by the current protocol should be critically monitored.

The analysis of polySia after IP from d_{132} cultures revealed a depletion of polySia-SynCAM 1 below the detection level, and a strong reduction of polySia-NCAM immunoreactivity (Fig. 3D). Western blot analysis of SynCAM in whole cell lysates of d_{132} cultures demonstrated that the broad SynCAM signal in the range of 110 kDa is still present, implicating a specific down-regulation of the polysialylated fraction of SynCAM 1 upon further differentiation (Fig. 3E). As polySia expression depends mainly on the balance between synthesis and degradation of polysialylated structures (Hildebrandt et al., 2010) we sought to assess if the down-regulation of polySia during oligodendrocyte maturation is a consequence of reduced polyST expression. As shown in Fig. 3F, a significant reduction of the ST8SIA2 mRNA level and a trend towards a reduction of ST8SIA4 was detected, suggesting regulation at the transcriptional level. In contrast to NCAM, polysialylation of SynCAM 1 is exclusively mediated by ST8SIA2 (Rollenhagen et al., 2012). Hence, the stronger reduction of ST8SIA2 mRNA is consistent with the more pronounced decrease of polySia-SynCAM 1. It will be of interest to further explore the mechanisms behind this transcriptional down-regulation, e.g., if it is mediated by miRNAs. So far, however, little is known about miRNA regulation of polySTs. As judged by the total number of predicted miRNA binding sites within the 3′UTR, both polySTs show a relatively high level of miRNA regulation (Kasper et al., 2014) and ST8SIA4 is a predicted target of miR-30b, which is abundant in primary human neuronal and glial cells and under-expressed in Parkinson's disease patients (Martins et al., 2011).

In summary, the current study demonstrates that human ESC-derived OPCs express polySia on the NCAM isoforms NCAM-180 and NCAM-140, and identifies SynCAM 1 as a novel polySia acceptor in human OPCs. So far, polySia has not been detected on OPCs of the human CNS, but on demyelinated axons and astrocytic cells in the vicinity of polySia-negative remyelinated axons of chronic multiple sclerosis lesions (Charles et al., 2002). Possibly, polysialylated OPCs were not found because OPCs are reduced and/or in a quiescent state in chronic multiple sclerosis lesions, which may prevent the generation of new myelin-forming oligodendrocytes (Wolswijk, 2002). Based on the broad evidence for a crucial role of polySia in OPC performance, the current finding that polySia-positive human OPCs readily produce polySia-negative, MBP-positive oligodendrocytes raises the expectation that polySia-positive OPCs provide a source for actively remyelinating cells in transplantation approaches for treating demyelinating diseases. Furthermore, considering the role of SynCAM 1 in the coordination of synaptic adhesion, the regulation of polySia-SynCAM 1 during oligodendrocyte maturation holds the potential to modulate synaptic contacts between NG2 cells/OPCs and axons and may be important for the onset of myelination. Since the polysialylation of SynCAM 1 depends exclusively on ST8SIA2 (Rollenhagen et al., 2012) the manipulation of ST8SIA2 activity should be considered as a strategy for guiding myelination from human stem cell-derived OPCs in biomedical applications. Indeed, ST8SIA2 may be an interesting druggable target to control polySia expression. Although further studies are needed to develop reagents such as specific small molecule inhibitors of polyST activity (Al Saraireh et al., 2013) or vectors for ST8SIA2 RNA interference (Brocco and Frasch, 2006), the time-controlled inhibition of ST8SIA2 after grafting of OPCs may offer a valuable tool in novel therapeutic approaches for, e.g., multiple sclerosis or spinal cord injury.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.03.001.

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