Sendai virus-mediated expression of reprogramming factors promotes plasticity of human neuroblastoma cells

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Neuroblastoma (NB) is the most common extracranial solid tumor that originates from multipotent neural crest cells. NB cell populations that express embryonic stem cell-associated genes have been identified and shown to retain a multipotent phenotype. However, whether somatic reprogramming of NB cells can produce similar stem-cell like populations is unknown. Here, we sought to reprogram NB cell lines using an integration-free Sendai virus vector system. Of four NB cell lines examined, only SH-IN cells formed induced pluripotent stem cell-like colonies (SH-IN 4F colonies) at approximately 6 weeks following transduction. These SH-IN 4F colonies were alkaline phosphatase-positive. Array comparative genomic hybridization analysis indicated identical genomic aberrations in the SH-IN 4F cells as in the parental cells. SH-IN 4F cells had the ability to differentiate into the three embryonic germ layers in vitro, but rather formed NBs in vivo. Furthermore, SH-IN 4F cells exhibited resistance to cisplatin treatment and differentiated into endothelial-like cells expressing CD31 in the presence of vascular endothelial growth factor. These results suggest that SH-IN 4F cells are partially reprogrammed NB cells, and could be a suitable model for investigating the plasticity of aggressive tumors.
particular stem cells give rise to diverse cell lineages, including Schwann cells, melanocytes, craniofacial cartilage, peripheral neurons, and glia. Some human NB cell lines partially retain a multipotent phenotype and can differentiate into several cell types. Intermediate type (I-type) NB cells possess morphological and biochemical properties of both neuroblastic and substrate-adherent NB cells, and differentiate into either type upon retinoic acid or BrdU treatment, respectively. I-type NB cell lines show increased tumorigenic potential compared with neuroblastic or substrate-adherent NB cells, suggesting that epigenetic changes in NBs contribute to aggressiveness. Indeed, recent studies have revealed that abnormal epigenetic regulation is one of the driving forces underlying NB tumorigenesis.

Somatic cell reprogramming by forced expression of defined transcription factors has paved the way to generate patient-specific stem cell sources. These induced pluripotent stem cells (iPSCs) possess key features of embryonic stem cells (ESC) including self-renewal and pluripotency. Several cancer cell lines have been reprogrammed via ectopic expression of defined transcription factors. Reprogrammed sarcoma and glioblastoma cells retain the same genomic aberrations as parental cells and can differentiate into multiple lineages. Reprogramming of cancer cells is a useful technique for investigating epigenetic mechanisms that affect the plasticity of tumor cells; this technique has not been reported in NB cells to date. Here, we attempted to reprogram human NB cells to promote plasticity using a Sendai virus (SeV) vector encoding Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC) for transgene-free reprogramming of iPSCs. We used this SeV vector system to reprogram NB cells to minimize the deleterious effects on the host genome and prevent subsequent gain of artificial tumorigenic activities.

Materials and methods

SeV-mediated expressions of reprogramming factors in NB cells. SeV vectors encoding the four Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC) were obtained from DNAVEC Corporation (Ibaraki, Japan) and used according to the manufacturer’s instructions. We plated 5 × 10⁵ cells and incubated them overnight in the appropriate medium. The next day, cells were transduced with SeV vectors at a multiplicity of infection of three and maintained at 37°C in 5% CO₂. At 24 h post-transduction, the virus-containing medium was removed and fresh medium was added every subsequent day. At 6 days post-transduction, cells were dissociated with Accutase (Stemcell Technologies, Vancouver, BC, Canada), counted, and re-plated onto mouse embryonic fibroblasts (MEFs). The next day, the media was switched to primate ES cell medium (ReproCELL) containing 4 ng/mL basic fibroblast growth factor (bFGF, ReproCell, Yokohama, Japan) and 10 μM ROCK inhibitor and incubated at 37°C in 3% CO₂. Medium was changed daily.

Endothelial tube formation assay. In vitro capillary-like tube formation was studied on Matrigel-coated wells in specific culture medium (Tube Formation Kit; Trevigen, Gaithersburg, MD, USA). NB cells were seeded onto matrigel-coated wells in Endothelial Basal Medium without serum in the presence of vascular endothelial growth factor (VEGF; 5–15 ng/mL) and bFGF (20–50 ng/mL). Normal human umbilical vein endothelial cells (HUVECs) maintained in Endothelial Cell Growth Medium 2 (PromoCell GmbH, Heidelberg, Germany) served as a positive control for tube formation. Tube-like structure formation on matrigel was observed over a 6–48 h period and results were recorded. To evaluate cell differentiation, NB cells were incubated in EndoGRO-MV-VEGF complete media kit (Millipore) with VEGF (5 ng/mL) on gelatin-coated plates. The medium was changed every other day for 1 week. Cells were then stained by immunofluorescence for CD31.

More detailed descriptions of the Material & Methods are provided in Suppl. Data S1.

Results

SeV-mediated expression of reprogramming factors in NB cells. High expression levels of pluripotency-associated genes in parental cells are related to the efficiency of iPSC generation. To identify suitable candidate cell lines for reprogramming, we analyzed the expression levels of pluripotency-associated genes—including NANO, OCT4, SOX2, and KLF4—in 24 NB cell lines. We selected the NB cell lines SK-N-AS and SK-N-DZ for reprogramming based on their high expression of two (NANO and KLF4) and three (NANO, OCT4, and KLF4) pluripotency-associated genes, respectively (Suppl. Fig. S1). We also selected the two I-type NB cell lines SH-IN and BE(2)-C, as they exhibit stem cell-like characteristics and express pluripotency-associated genes at high levels (Suppl. Figs S1B and S2).

In contrast to BJ cells, all four NB cell lines proliferated rapidly in serum-free ES medium, with MEF cells dying within a week. Therefore, we prepared new MEF feeder cells weekly. SH-IN cells gave rise to iPSC-like colonies (SH-IN 4F) approximately 6 weeks after transduction (Fig. 1 and Suppl. Fig. S3). Each colony was then transferred into two 96-well plates using a transfer pipet. One plate was used for AP staining and the other for passage. We selected 12 clones based on strong AP activity and cultured them for 2 months to eliminate the SeV transgenes. We were unable to detect any colonies from the SK-N-AS, BE(2)-C, or SK-N-DZ cell lines up to 7 weeks after induction. The SH-IN 4F cells could not be expanded and maintained in culture for at least 40 passages. To determine the presence of transgenes or a viral backbone, we examined clones using reverse transcription-polymerase chain reaction (RT-PCR). We selected three clones (2, 7, and 11) based on eliminated transgenes (Fig. 1b). SH-IN 4F cells were positive for AP staining (Fig. 1c). Some cells at the periphery of SH-IN 4F colonies lost AP activity 3 days after passage. Therefore, SH-IN 4F cells were passaged every 3 days.

Characterization of SH-IN 4F cells. Immunocytochemistry analysis showed that SH-IN 4F cells expressed ESC-related core transcription factors, including NANO, OCT4, and SOX2 in a manner similar to neonatal human foreskin fibroblast BJ-iPSCs and human dermal fibroblast-derived iPSCs (201B7; Fig. 2a and Suppl. Fig. S4). Expression of ESC-specific surface markers, including stage specific embryonic antigen-4 (SSEA-4), tumor related antigen-1 (TRA-1-60), and tumor related antigen-1-81 (TRA-1-81), was also apparent in SH-IN 4F cells (Fig. 2a). RT-PCR analysis revealed that expression of endogenous OCT4, SOX2, and KLF4 was induced in SH-IN 4F cells at levels comparable with those in iPSCs (Suppl. Fig. S5). NANO, c-MYC, LIN28, and βTERT were highly expressed in SH-IN 4F cells compared with iPSCs, whereas CHD1, DNMT3B, and TGFβT were not induced (Suppl. Fig. S5).

Reprogramming of somatic cells is accompanied by demethylation of the promoter regions of key pluripotency-associated transcription factors. We used bisulfite genomic
Fig. 1. Transgene-free reprogramming of SH-IN cells. (a) Schematic outlining the transgene-free reprogramming of SH-IN cells using a SeV vector. SH-IN cells were infected with the SeV vector encoding the transcription factors OCT4, SOX2, KLF4, and c-MYC. (b) Total RNA was extracted from cells at 6 days to 4 months post-transduction and analyzed by semi-quantitative RT-PCR to verify transgene elimination. (c) Typical morphology of parental SH-IN and foreskin fibroblast BJ cells (left panels). Formation of induced pluripotent stem cells (iPSCs)-like colonies after transduction (right panels). SH-IN 4F cells stained positive for alkaline phosphatase (AP, bottom row). Scale bar: 100 μm.
SH-IN 4F cells express high levels of pluripotency-associated genes. (a) SH-IN 4F cells (clone 2) expressed undifferentiated embryonic stem cell (ESC) markers and surface antigens (NANOG, OCT4, SOX2, SSEA-4, TRA-1-60, and TRA-1-81) as determined by immunocytochemical analysis. Nuclei were stained with DAPI (blue). Results are representative of three independent experiments. Scale bar: 75 μm. (b) Epigenetic modification of pluripotency-related genes was examined by bisulfite genomic sequencing. OCT4 was repressed in EBs from 201B7-iPS cells, and this was accompanied by the induction of βIII tubulin (Suppl. Fig. S9C). However, expression of OCT4 was repressed in EBs from 201B7-iPS cells, and this was accompanied by the induction of βIII tubulin (Suppl. Fig. S9C). In contrast, SH-IN parental cells exhibited homogeneous staining of βIII tubulin and lower expression of OCT4 (Suppl. Fig. S9D). Most cells in SH-IN 4F colonies were OCT4-positive, with some of the peripheral cells staining positive for βIII tubulin (Suppl. Fig. S9D). At more than 3 days after passage, OCT4 localized to both the nucleus and cytoplasm of SH-IN 4F cells in colonies (Suppl. Fig. S9D). However, similar to human iPSCs (Suppl. Fig. S4), OCT4 mainly localized to the nuclei of SH-IN 4F cells when cells were passaged every 3 days (Fig. 2a). Therefore, spontaneous differentiation of SH-IN 4F cells changed the subcellular localization of OCT4.

SH-IN 4F cells differentiate into vascular endothelial-like cells. OCT4 is expressed in a subpopulation of NB cells, and OCT4-positive NB cells serve as progenitors of tumor-derived endothelial cells. We therefore examined the ability of SH-IN 4F cells to form vascular endothelial-like structures. Normal HUVECs were used as a positive control. SH-IN 4F cells formed a network of cells (Fig. 5a, center panel), although the tube networks were thick and less organized compared with HUVECs (Fig. 5a, left panel). Parental SH-IN cells gathered together under the differentiating medium without forming tube-like structures (Fig. 5a, right panel). SH-IN 4F cells expressed the endothelial-specific marker CD31 (Fig. 5b) and the proportion of CD31+ cells was significantly increased compared with parental SH-IN cells (Fig. 5c).

We next performed immunohistochemical staining for human CD31 (Suppl. Fig. S10A,B) and the immature endothelial cell marker prostate-specific membrane antigen (PSMA; Suppl. Fig. S10C,D), using tumor xenografts. Some spindle-shape cells were positive for human PSMA, but no specific signals for human CD31 were detected in the xenograft tumors, although western blots for CD31 suggested the induced expression of CD31 in SH-IN 4F tumors (Suppl. Fig. S10E). These results suggest that both SH-IN 4F and SH-IN cells have limited capacity for endothelial differentiation in vivo. The number of PSMA-positive cells in SH-IN 4F tumors was not significantly different from that in SH-IN tumors.

SH-IN 4F cells have a chemoresistant phenotype. We next treated SH-IN and SH-IN 4F cells with CDDP and evaluated apoptotic cell death by TUNEL assays. Apoptosis induced by CDDP was significantly reduced in SH-IN 4F cells compared with SH-IN cells (Fig. 6). We then investigated the molecular mechanisms underlying chemoresistance in SH-IN 4F cells by measuring the expression of several genes known to promote chemoresistance in NB. Increased expression of CD133, ALDH1A1, ARID3B, and NCIYM was detected in SH-IN 4F cells, while MYCN expression did not change (Suppl. Fig. S11). In contrast, reprogramming of BJ cells
Fig. 3. SH-IN 4F cells possess similar genomic alterations as parental SH-IN cells. Array comparative genomic hybridization. Panels show chromosomal gains and losses in parental SH-IN (a) and SH-IN 4F clones 2, 7, and 11 (b–d). A value of zero (0) indicates no loss or gain of chromosome, whereas + and − indicate gain and loss of copy number for each genomic location, respectively. SH-IN parental cells (a) exhibited ‘mixed’ genomic alterations, including gains of chromosomes 1p, 1q, 2p, 7, 9q, and 17q, and losses of 14q, 16q, and 22q. Similar genomic alterations were observed in the SH-IN 4F colonies (b–d).
Fig. 4. Embryoid body (EB)-mediated differentiation of SH-IN 4F cells. (a) Representative phase contrast images of EBs generated from SH-IN 4F cells. Undifferentiated cells at day 0 and differentiated EBs at days 2, 7, and 10. Scale bar: 100 μm. (b) Expression of endodermal (PAX6 and SOX1), mesodermal (HAND1 and FOXF1) and endodermal (AFP and GATA6) markers were examined by qPCR. The x-axis represents relative fold induction compared with day 0. All values were normalized to β-actin mRNA expression levels. (c) Immunofluorescence analysis of SH-IN 4F (clone 2) after EB differentiation: endoderm (GATA6), mesoderm (α-smooth muscle actin; α-SMA) and ectoderm (βIII tubulin). Nuclei were stained with DAPI (blue). Scale bars: 75 μm (GATA6 and α-SMA) and 50 μm (βIII tubulin). Results are representative of three independent experiments.

Fig. 5. Endothelial tube formation by SH-IN 4F cells. (a) SH-IN 4F cells cultured in serum-free medium supplemented with basic fibroblast growth factor (bFGF) and EGF to examine plasticity. Representative micrograph of the tube network formed by SH-IN 4F cells and HUVEC cells. Scale bar: 300 μm. (B) HUVEC cells or SH-IN cells were cultured in differentiating medium supplemented with 2% FCS and vascular endothelial growth factor (VEGF for 7 or 10 days, respectively. Expression of the endothelial-specific marker CD31 was detected by immunofluorescence. Scale bar: 50 μm. Nuclei are stained with DAPI (blue). (c) Quantification of CD31-expressing cells as measured by immunofluorescence. Columns represent mean values from three replicate experiments, error bars represent SD. Results are representative of three independent experiments performed using clone 7.

induced expression of MYCN, NCYM, and ARID3B (Suppl. Fig. S11C).

Discussion

We expressed reprogramming factors in NB cells using a non-integrating SeV vector. Similar to human iPSCs, SH-IN 4F cells expressed pluripotency-associated genes following reprogramming. Furthermore, markers of all three embryonic germ layers were induced during EB formation. Some cells at the periphery of SH-IN 4F colonies spontaneously differentiated into neuronal cells, with cells positive for both OCT4 and βIII tubulin detected. OCT4-positive NB cells possess the ability to differentiate into tumor cell-derived vascular-endothelial cells, and OCT4 is highly expressed in stem-like cells—such as side-population cells. Consistent with these reports, SH-IN 4F cells formed vascular endothelial-like structures in differentiating medium, and expressed the endothelial marker CD31. Taken together these findings indicate that SeV-mediated expression of reprogramming factors increased the plasticity of SH-IN cells.

Reprogramming of SH-IN cells was not sufficient to allow endothelial differentiation in vivo, though human CD31 expression was slightly induced. Given that the percentage of cells positive for PSMA in parental SH-IN cells was relatively low compared with other NB cell lines, endothelial differentiation of SH-IN cells in vivo may also be rare. Further experiments are required to clarify the molecular mechanisms underlining the barriers to endothelial differentiation of SH-IN cells.

Demethylation of the OCT4 promoter was limited in SH-IN 4F cells, although the expression level of OCT4 mRNA was comparable with that in iPSCs. Additionally, there was no induction of CHD1, DMNT3B, or TDGF1 expression in SH-IN 4F cells, and endogenous expression of reprogramming factors was minimally downregulated during EB formation. GATA6 localized to the cytoplasm in SH-IN 4F-derived EBs, and SH-IN 4F cells formed NBs—but not teratomas—in vivo. These results suggest that SH-IN 4F cells, while de-differentiated, were not fully reprogrammed.

Similar to SH-IN 4F cells, reprogrammed A549 human lung cancer cells highly expressed OCT4 with limited demethylation of the OCT4 promoter. Reprogrammed A549 cells generated invasive tumors compared with the parental cells, but the tumors were not typical teratomas, suggesting that the partial de-differentiation induced by reprogramming contributes to tumor aggressiveness. Using in vivo reprogramming mouse models, Ohnishi et al. recently reported that premature termination of reprogramming resulted in development of childhood blastomas—but not teratomas. Meanwhile long-term in vivo activation of Yamanaka factors facilitated teratoma formation. Ikegaki et al. revealed that treatment of NB cell lines with epigenetic modifiers in sphere-forming cultures enabled...
re-activation of reprogramming factors and induced development of poorly differentiated stem cell-like NB cells. Injection of these induced cancer stem cells into mice resulted in the formation of NBs—not teratomas—supporting the notion that de-differentiation by partial reprogramming promotes tumor aggressiveness.

Our findings reveal that partial reprogramming of SH-IN cells facilitates CDDP resistance and enhanced differentiation into endothelial-like cells, resulting in NB heterogeneity in vitro. Therefore, de-differentiation of SH-IN cells by partial reprogramming may model the plasticity of tumor cells in NBs. Additionally, expression of CD133, ALDH1A1, ARID3B, and NCYM—but not MYCN—was induced in SH-IN 4F cells. Previous reports suggest that the expression level of MYCN is not associated with poor prognosis in MYCN non-amplified NBs, while the level of NCYM is associated with poor prognosis. Therefore, overexpression of reprogramming factors may contribute to chemoresistance in MYCN-non amplified SH-IN cells, possibly by induction of these stem-cell related genes.

Our present findings indicate that partially reprogrammed NB cells could be a valuable in vitro model for understanding how NB cells maintain plasticity and aggressiveness.

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Disclosure Statement
Yasuii Ueda is an employee of DNAVEC Corporation. This does not alter the authors’ adherence to all Cancer Science policies. There are no other relevant declarations relating to employment, consultancy, patents, or products in development or marketed products.

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Supporting Information
Additional supporting information may be found in the online version of this article:
Data S1. More detailed descriptions of the Material & Methods.
Fig. S1. Expression levels of pluripotency-associated genes in 24 human NB cell lines.
Fig. S2. I-type NB cells highly express pluripotency-associated genes.
Fig. S3. SH-IN 4F cells disperse over the culture surface and form monolayers.
Fig. S4. Generation of iPSCs from BJ cells by SeV vectors.
Fig. S5. Expression of pluripotency-associated genes in SH-IN 4F cells.

Abbreviations
CDDP Cis-diamminedichloroplatinum
iPSCs Induced pluripotent stem cells
NB Neuroblastoma
SeV Sendai virus
Fig. S6. EB-mediated differentiation of human iPSCs.
Fig. S7. SH-IN 4F cells retain expression of pluripotency-associated gene during EB formation.
Fig. S8. SH-IN 4F cells formed neuroblastomas in NOD-SCID mice.
Fig. S9. SH-IN 4F cells spontaneously differentiate towards neuronal lineages.
Fig. S10. Expression of CD31 and PSMA protein in SH-IN 4F tumors analyzed by immunohistochemistry.
Fig. S11. Expression levels of genes related to stemness or chemoresistance in SH-IN 4F cells.