Differentiation, polarization, and migration of human induced pluripotent stem cell-derived neural progenitor cells co-cultured with a human glial cell line with radial glial-like characteristics

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ABSTRACT

Here we established a unique human glial cell line, GDC90, derived from a human glioma and demonstrated its utility as a glial scaffold for the polarization and differentiation of human induced pluripotent stem cell-derived neural progenitor cells (iPSC-NPCs). When co-cultured with GDC90 cells, iPSC-NPCs underwent rapid polarization and neurite extension along the radially spreading processes of the GDC90 cells, and showed migratory behavior. This method is potentially useful for detailed examination of neurites or for controlling neurites behavior for regenerative medicine.

KEY WORDS

Induced pluripotent stem cells, Glioma, Neuronal migration, Radial glia, PiggyBac transposon,

Regenerative medicine

ABBREVIATIONS

iPSCs, induced pluripotent stem cells; iPSC-NPCs, iPSC-derived neural progenitor cells; RG,

radial glia; PB, PiggyBac

INTRODUCTION

During embryonic corticogenesis, neural progenitor cells (NPCs) and neuronal cells have complicated interactions with glial cells. In particular, radial glia (RG) cells construct the framework and control the alignment of neuronal cells during embryonic CNS development [1]. Glial cells are also important for the differentiation of NPCs derived from pluripotent stem cells (PSCs) and induced pluripotent stem cells (iPSCs) [2]. It is now possible to investigate developmental processes of the human central nervous system (CNS) and the pathogenesis of neural disorders using human PSCs [3-6]. iPSC-derived NPCs (iPSC-NPCs) can produce both neuronal and glial cells; however, they tend to remain neurogenic for a prolonged period [7,8]. Extensive research has led to methods for promoting iPSC differentiation toward specific lineages. However, the differentiation propensities of the iPSCs and iPSC-NPCs are not completely controlled. Furthermore, iPSC-NPCs produce neuronal cells with disordered and randomly arranged neurites, which complicates the examination of neurite morphology and dynamics. The disorderly arrangement of neurites in *in vitro* cultures may be due to the absence of the radial glial scaffold that is abundant during *in vivo* corticogenesis.

This shortage of glial scaffolds *in vitro* is addressed by co-culturing iPSC-NPCs with glial

cells. Human iPSC-NPCs can be induced to develop into mature neurons by culturing them with mouse primary astrocytes [2]. However, the isolation of primary glial cells for each experiment is time-consuming and laborious. Thus, the use of a human glial cell line as a scaffold for iPSC-NPC differentiation may be a more efficient approach.

Here, we established a co-culture system using iPSC-NPCs and a unique glial cell line, GDC90, which promoted iPSC-NPC differentiation, polarization, and migration. Use of this unique human glial cell line may shed light on the detailed morphological behaviors of iPSC-NPCs and contribute to their application in regenerative medicine.

MATERIALS AND METHODS

Human tissues and cells

This study was carried out in accordance with the principles of the Helsinki Declaration, and approval for the use of human tissues and cells was obtained from the ethical committees of Osaka National Hospital and Keio University School of Medicine (No.94, 110, 146, IRB0713). Surgically removed primary brain tumor tissues were collected at the Osaka National Hospital with written informed consent.

Human iPSC clone 201B7 [9], obtained from the RIKEN cell bank (Tsukuba, Japan), was propagated on Mitomycin C-treated mouse embryonic fibroblast (MEF) feeder cells as previously described [10].

Two human glioma cell lines derived from glioblastoma multiforme (GBM), U-251MG (IFO50288, The Japan Health Sciences Foundation, Tokyo, Japan) and U-87MG (HTB-14, ATCC), were propagated in culture medium with 10% fetal bovine serum. Human neural stem cells (oh-NSC-3-fb) derived from the fetal forebrain were propagated by the neurosphere method as previously described [11].

Isolation of glioma-derived cells

Glioma-derived cells (GDCs) were isolated from GBM specimens using a serum-free suspension culture method as previously described [11].

Generation of iPSC-NPCs

NPCs were generated from the human iPSC line 201B7 by embryoid body formation and subsequent neurosphere culture as previously described [8].

Genetic labeling by PiggyBac (PB) transposon-mediated gene transfer

A CAG promoter [12]-driven expression cassette and a neomycin-resistance gene cassette

from pEBmulti-neo (Wako, Osaka, Japan) were cloned into pPB533A2 (System Biosciences, CA, USA), replacing components between two core insulators and the PB terminal repeat sequences. The coding sequence for EGFP or for tdTomato [from ptdTomato-N1 (Clontech, CA, USA)] was cloned downstream of the CAG promoter to construct pPB-CAG-EGFP (or -tdTomato) -Neo using restriction enzymes.

Nucleofection of GDC90 cells and iPSC-NPCs was performed as previously described [13]. Both 7.5 µg of the fluorescent protein expression vector and 2.5 µg of the PB transposase expression vector PB200A (System Biosciences) were introduced into $3\text{-}5\times 10^6$ cells. Fluorescent proteins were expressed in U-87MG and U-251MG cells by transfecting them with PB vectors using the FuGene HD Transfection Reagent (Promega, WI, USA). Geneticin (200-600 µg/ml, Life Technologies) was maintained in the medium until the second passage.

Quantitative RT-PCR

Total RNAs were isolated using the RNeasy Mini Kit (Qiagen, Venulo, Netherlands). Template cDNA samples were synthesized using the Prime Script RT Reagent kit (Takara, Shiga, Japan). Real-time quantitative PCR was performed with the PowerSYBR Green PCR Mix (Life

Technologies) and the ABI7300 Real-time PCR system (Life Technologies) using gene-specific primer pairs (Supplemental Table 1). The expression value (Ct value) of each gene was normalized to that of GAPDH, and the normalized expression values were compared as previously described [10].

Immunocytochemistry and imaging analysis

GDC90 or iPSC-NPC aggregates were plated on Matrigel-GFR- (BD Biosciences, NJ, USA) coated glass chamber slides (Matsunami Glass, Osaka, Japan). The samples fixed with 4% paraformaldehyde were incubated with primary antibody overnight at 4°C and then in secondary antibody and the DRAQ5 nuclear stain (Biostatus, Leicestershire, UK) for 1 hour at room temperature. Images were acquired using LSM510 and LSM5 software (Carl Zeiss, Oberkochen, Germany).

The primary antibodies were rabbit anti-Nestin (1:500) [14], rabbit anti-glial fibrillary acidic protein (GFAP) (1:80, Sigma), mouse anti- β III tubulin (1:500, Covance, Princeton, NJ, USA), rabbit anti-brain lipid binding protein (BLBP) (1:500, Millipore), rabbit anti-SOX2 (1:500, Chemicon), and rabbit anti-doublecortin (DCX) (1:400, Cell Signaling, Danvers, MA, USA). The secondary antibodies were goat anti-rabbit IgG Alexa Fluor® 488 (1:1,000, Molecular

Probes, CA, USA), goat anti-mouse IgG Alexa Fluor® 648 (1:1,000, Molecular Probes), and goat anti-rabbit IgG Alexa Fluor® 648 (1:1,000, Molecular Probes). Fluorescence microscopy was performed with an IX81 inverted microscope (Olympus, Tokyo, Japan). Time-lapse images were taken in a DH35i micro-incubation system with an automatic temperature controller (Warner Instruments, CT), using Lumina Vision software (Mitani, Fukui, Japan).

iPSC-NPC and GDC90 co-culture

For adjacent co-cultures, approximately 10 spheres each of GDC90 cells and iPSC-NPCs were collected and plated on Matrigel-GFR- (BD Biosciences) coated glass-bottom dishes (Matsunami Glass) in differentiation medium containing Neurobasal Medium (Life Technologies), 2% B27 supplement (Life Technologies), 2 mM L-glutamine (Life Technologies), and 1% Antibiotic-Antimycotic (Life Technologies). For adjacent co-cultures using U-87MG or U-251MG cells, aggregates were formed from 5×10^3 cells in low-attachment 96-well V-bottom (conical) plates (PrimeSurface, Sumitomo Bakelite, Tokyo, Japan). For mixed co-cultures, 5×10^3 U-87MG or U-251MG cells or 5.0×10^3 GDC90 cells and 5.0×10^3 iPSC-NPCs were seeded into a single well of a low attachment 96-well V-bottom plate one day before plating on the Matrigel-GFR-coated dishes.

Three-dimensional co-culture of iPSC-NPCs and GDC90 cells on a silicate membrane

Mixed aggregates containing 1×10^4 cells each of iPSC-NPCs and GDC90 cells, generated as described above, were plated on a CellBed silicate fiber membrane (Japan Vilene, Tokyo, Japan, <http://www.cellbed-jp.com/>). The green fluorescence of neurites extending from the EGFP-expressing iPSC-NPCs was observed with an IX81 fluorescence microscope.

Statistical analysis

All data represent the mean of values determined from three experiments. The statistical analysis of the results was performed by Welch's t-test.

RESULTS

Isolation and establishment of fluorescently labeled human GDCs

We isolated a cell line, GDC90, from the tumor specimen of a 74-year-old female glioblastoma multiforme (GBM) patient. To distinguish GDC90 cells from host or co-cultured cells, we used the PB transposon vector which contains a tdTomato-expressing cassette driven by the CAG promoter, a neomycin-resistance gene cassette, and the PB terminal repeats sequence (Figure 1A). Following nucleofection of this vector and neomycin selection, GDC90

cells were isolated that stably expressed tdTomato and maintained fluorescence for over 10 passages, even when antibiotic selection was discontinued (Figure 1B, C). Glioma cell lines (U-87MG, U-251MG), and GDC40 cells, another GDC line isolated from a GBM patient (Okada et al, submitted), were also labeled using the same system (Figure 1D-I). To observe the dynamic behavior of iPSC-NPCs, we established EGFP-labeled iPSC-NPC cell lines using the same nucleofection method (Figure 1J, K).

To characterize the glial cell lines, we examined neural marker expression using quantitative RT-PCR and immunocytochemistry. Quantitative RT-PCR (Figure 1Q) showed that the Nestin mRNA level was higher in GDC90 than in the other glial cell lines ($n=3$, $p<0.001$) and that GFAP was more highly expressed in GDC90 and U-251MG cells than in iPSC-NPCs ($n=3$, $P<0.001$). The BLBP expression was higher in GDC90 and GDC40 than in U-251MG cells ($n=3$, $P<0.001$, $P<0.01$ respectively), where high BLBP expression was previously reported [15]. The PAX6 expression was higher in GDC90 than GDC40 cells ($n=3$, $P<0.01$), but lower than in iPSC-NPCs ($n=3$, $P<0.01$). Immunocytochemical analyses showed that plated GDC90 cells had a polarized morphology with extended processes that were Nestin-, GFAP-, and BLBP-positive (Figure 1L-N), and SOX2-positive nuclei (Figure 1O).

Collectively, these results showed that GDC90 cells exhibited a molecular expression profile that was characteristic of both glial and NPCs. This profile may be reminiscent of RG cells, which are one of the candidate originators of human gliomas.

iPSC-NPC and glial cell co-culture system

To assess the behavior of iPSC-NPCs in contact with glial cell lines, we performed two different co-culture experiments. In the first, we plated a fluorescently labeled glial cell line (U-251MG, U-87MG, or GDC90) and iPSC-NPC spheres on the same dish (Figure 2A). GDC90 aggregates plated on the dish extended their tdTomato-positive fibers in a regular and radial manner, and the neighboring iPSC-NPCs extended their EGFP-positive fibers along the GDC90 radial fibers (Figure 2B). Most of the iPSC-NPC processes extended into the core of the adjacent GDC90 aggregate (Figure 2C), and some of them even passed through the core and exited along the radial fiber on the other side (Supplementary movie 1). In contrast to GDC90, U-251MG (Figure 2D) and U-87MG (Figure 2E) processes did not spread radially from the aggregates, and neighboring iPSC-NPCs did not extend their processes in a regular and radial manner in the co-culture.

In the second experiment, we generated mixed aggregates from fluorescently labeled glial

cells and iPSC-NPCs (Figure 2F). Soon after plating, numerous iPSC-NPCs had migrated out from the sphere along the radial fibers of GDC90 cells (Figure 2G, H). Approximately 10 days after plating, we observed the translocation-like migration of iPSC-NPC-derived neurons along the radial fibers of GDC90 cells (Supplementary movie 2). The U-251MG and U-87MG lines did not exhibit these scaffolding properties, possibly because they were isolated and cultured as adherent cells in serum-containing medium, rather than as floating neurospheres in serum-free medium. We therefore tested another human glioma-derived cell line, GDC40, which was isolated using the serum-free neurosphere method. Interestingly, although the GDC40 cells expressed radial glial marker BLBP and Nestin, similar to GDC90 cells (Figure 1Q), they did not efficiently support iPSC-NPC neurites extension (Figure 2I, J).

Immunocytochemical analyses of the iPSC-NPC-GDC90 co-culture showed that the EGFP-positive fibers extending from the iPSC-NPCs were β III tubulin-positive neurites (Figure 2K, L), which also expressed DCX, a characteristic of migrating neurons (Figure 2M). DCX-positive iPSC-NPC-derived neurons were observed in association with tdTomato-positive glial fibers (Figure 2N), suggesting that the GD90-co-cultured iPSC-NPCs became polarized and migrated along GDC90 processes, similar to their movement along glial scaffolds *in vivo*.

In both cultures, the iPSC-NPCs differentiated into neurons that migrated along GDC90-derived radial fibers, which supported both afferent and efferent neurite extension. Thus, GDC90 represents a unique glial cell line that supports iPSC-NPC differentiation, polarization, and migration.

Glioma-derived glial cells exhibit a strong affinity for iPSC-NPCs.

In the co-culture system, iPSC-NPCs showed different responses to monolayer-cultured glioma-derived cells (U-251MG and U-87MG) versus neurosphere-cultured-GDCs (GDC90). We considered that the differently prepared GDCs might have different affinities for iPSC-NPCs. To explore this possibility, we generated mixed aggregates that contained equal numbers of glioma-derived cells and iPSC-NPCs. As expected, the GDC90 cells and iPSC-NPCs formed single aggregates, in which iPSC-NPCs were relatively abundant in the inner portion (Figure 3D). In contrast, iPSC-NPCs and U-251MG or U-87MG failed to form homogeneous aggregates (Figure 3E, F). Unexpectedly, the iPSC-NPCs formed patchy clusters in these aggregates after one day of culture, and the two cell types formed completely separate aggregates after five days (Figure 3H, I). These observations indicated that GDC90 cells might have a higher affinity for iPSC-NPCs than do U-251MG or U-87MG cells. This may be partly

due to differences in cell adhesion molecule expression. In fact, quantitative RT-PCR showed that the N-cadherin expression was similar in GDC90 cells and iPSC-NPCs ($P=0.3$), but approximately half that level in U-251MG cells ($P<0.01$) (Figure 3J). Thus, the high affinity of GDC90 and iPSC-NPCs for each other might reflect their shared expression of N-cadherin, which is abundant in neural tissue.

GDC90 and iPSC-NPC co-culture on an artificial membrane.

RG cells provide a structural framework for embryonic corticogenesis and are necessary for CNS regeneration in lower animals. Thus, we further explored the effects of GDC90 cells on iPSC-NPC behavior. To induce neurite extension three-dimensionally from iPSC-NPCs, we plated mixed GDC90 and iPSC-NPC aggregates on a silicate fiber substrate. In these membranes, large slits between the silicate fibers represent sites where GDC90 cells and iPSC-NPCs can interact directly. When these cell types were co-cultured on the artificial substrate, GDC90 cells spread across the surface of the membrane, and their outgrowth led to scaffold formation, followed by iPSC-NPC-derived neurite extension (Figure 4A-C). Even after two months of culture, the iPSC-NPC-derived neurons survived on the membrane; they exhibited a highly polarized morphology, characterized by the extension of long neurites (Figure

4D-F). Based on these observations, we concluded that GDC90 cells could be used in conjunction with the artificial substrate as a three-dimensional scaffold for iPSC-NPC differentiation.

DISCUSSION

Here, we established a unique human glial cell line, GDC90, which exhibited a radial glial-like molecular signature, a polarized shape, and radially spreading processes. Compared with the other glial cell lines tested, GDC90 cells showed a higher affinity for iPSC-NPCs, consistent with a previous report indicating that various glioma cell lines have different affinities for NSCs [16]. Consistent with this higher affinity, GDC90 cells expressed more N-cadherin than the other glioma-derived cell lines. Considering that during mouse corticogenesis, N-cadherin is essential for the attachment between radial glial cells and migrating neurons [17], N-cadherin may contribute to the scaffolding function of GDC90 cells in our *in vitro* system. The molecular signature and scaffolding properties of the GDC90 cells suggest they share some characteristics with RG cells. Our findings support the possibility that RG cells are involved in human glioma development [18-20].

Co-culture of the fluorescently labeled cells showed that GDC90 cells could support the differentiation, polarization, and migration of iPSC-NPCs. Our findings suggest that the co-culture of RG-like GDC90 cells converts iPSC-NPCs to polarized neurons that migrate *in vitro*, consistent with a previous report showing that mouse neuronal migration *in vitro* required the presence of glial cells, which were prepared from fetal mouse brains or cultured neural stem cells [21,22]. Thus, our unique co-culture system enables *in vitro* modeling of the differentiation, polarization, and migration of iPSC-NPCs without *in vivo* xeno-transplantation procedures.

Non-vertebrate or lower vertebrate animals can reconstruct a functional CNS via RG cell activation prior to neurogenesis [23,24,25]. Here, we attempted to recapitulate this regenerative process on a three-dimensional scaffold. The co-culture of GDC90 cells and iPSC-NPCs on a silicate fiber membrane supported the extension of neurites from the iPSC-NPCs and the development of iPSC-NPC-derived polarized neurons that were capable of long-term survival. This membrane is so easy to handle that it may be technically possible to transplant this well-differentiated neuronal tissue. Conceptually, “glial-controlled regeneration” might provide a hint for regeneration of injured CNS. Although clinical applications will require the replacement of tumor-derived glial cells by iPSC-derived RGs [26, 27], GDC90 or mitotically

inactivated GDC90 cells will still be useful for basic research until advances in differentiation methods enable the reproducible, stable and abundant production of iPSC-derived RG cells.

CONCLUSION

We established a unique human glial cell line, GDC90, and examined its effects on iPSC-NPC differentiation, polarization, and migration. This simple technique has the potential to accelerate the research and development of iPSC-based disease modeling and regenerative medicine.

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REFERENCES

- [1] P. Rakic, Evolution of the neocortex: a perspective from developmental biology, *Nat Rev Neurosci* 10 (2009) 724-735.
- [2] X. Tang, L. Zhou, A.M. Wagner, M.C. Marchetto, A.R. Muotri, F.H. Gage, G. Chen, Astroglial cells regulate the developmental timeline of human neurons differentiated from induced pluripotent stem cells, *Stem Cell Res* 11 (2013) 743-757.
- [3] I. Espuny-Camacho, K.A. Michelsen, D. Gall, D. Linaro, A. Hasche, J. Bonnefont, C. Bali, D. Orduz, A. Bilheu, A. Herpoel, N. Lambert, N. Gaspard, S. Peron, S.N. Schiffmann, M. Giugliano, A. Gaillard, P. Vanderhaeghen, Pyramidal neurons derived from human pluripotent stem cells integrate efficiently into mouse brain circuits in vivo, *Neuron* 77 (2013) 440-456.
- [4] M. Eiraku, K. Watanabe, M. Matsuo-Takasaki, M. Kawada, S. Yonemura, M. Matsumura, T. Wataya, A. Nishiyama, K. Muguruma, Y. Sasai, Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals, *Cell Stem Cell* 3 (2008) 519-532.
- [5] Y. Shi, P. Kirwan, J. Smith, H.P. Robinson, F.J. Livesey, Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses, *Nat Neurosci* 15 (2012) 477-486,

S471.

[6] M.A. Lancaster, M. Renner, C.A. Martin, D. Wenzel, L.S. Bicknell, M.E. Hurles, T. Homfray, J.M. Penninger, A.P. Jackson, J.A. Knoblich, Cerebral organoids model human brain development and microcephaly, *Nature* 501 (2013) 373-379.

[7] A. Falk, P. Koch, J. Kesavan, Y. Takashima, J. Ladewig, M. Alexander, O. Wiskow, J. Taylor, M. Trotter, S. Pollard, A. Smith, O. Brustle, Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons, *PLoS One* 7 (2012) e29597.

[8] Y. Imaizumi, Y. Okada, W. Akamatsu, M. Koike, N. Kuzumaki, H. Hayakawa, T. Nihira, T. Kobayashi, M. Ohyama, S. Sato, M. Takanashi, M. Funayama, A. Hirayama, T. Soga, T. Hishiki, M. Suematsu, T. Yagi, D. Ito, A. Kosakai, K. Hayashi, M. Shouji, A. Nakanishi, N. Suzuki, Y. Mizuno, N. Mizushima, M. Amagai, Y. Uchiyama, H. Mochizuki, N. Hattori, H. Okano, Mitochondrial dysfunction associated with increased oxidative stress and alpha-synuclein accumulation in PARK2 iPSC-derived neurons and postmortem brain tissue, *Mol Brain* 5 (2012) 35.

[9] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka,

Induction of pluripotent stem cells from adult human fibroblasts by defined factors, *Cell* 131

(2007) 861-872.

[10] T. Shofuda, D. Kanematsu, H. Fukusumi, A. Yamamoto, Y. Bamba, S. Yoshitatsu, H.

Suemizu, M. Nakamura, Y. Sugimoto, M.K. Furue, A. Kohara, W. Akamatsu, Y. Okada, H.

Okano, M. Yamasaki, Y. Kanemura, Human Decidua-Derived Mesenchymal Cells are a

Promising Source for the Generation and Cell Banking of Human Induced Pluripotent Stem

Cells, *Cell Med* 4 (2013) 125-147.

[11] Y. Kanemura, H. Mori, S. Kobayashi, O. Islam, E. Kodama, A. Yamamoto, Y. Nakanishi, N.

Arita, M. Yamasaki, H. Okano, M. Hara, J. Miyake, Evaluation of in vitro proliferative activity

of human fetal neural stem/progenitor cells using indirect measurements of viable cells based on

cellular metabolic activity, *J Neurosci Res* 69 (2002) 869-879.

[12] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants

with a novel eukaryotic vector, *Gene* 108 (1991) 193-199.

[13] S. Marchenko and L. Flanagan, Transfecting human neural stem cells with the Amaxa

Nucleofector. *J Vis Exp* 6 (2007) 240.

[14] Y. Nakamura, M. Yamamoto, E. Oda, A. Yamamoto, Y. Kanemura, M. Hara, A. Suzuki, M.

- Yamasaki, H. Okano, Expression of tubulin beta II in neural stem/progenitor cells and radial fibers during human fetal brain development, *Lab Invest* 83 (2003) 479-489.
- [15] A. De Rosa, S. Pellegatta, M. Rossi, P. Tunici, L. Magnoni, M.C. Speranza, F. Malusa, V. Miragliotta, E. Mori, G. Finocchiaro, A. Bakker, A radial glia gene marker, fatty acid binding protein 7 (FABP7), is involved in proliferation and invasion of glioblastoma cells, *PLoS One* 7 (2012) e52113.
- [16] O. Heese, A. Disko, D. Zirkel, M. Westphal, K. Lamszus, Neural stem cell migration toward gliomas in vitro, *Neuro Oncol* 7 (2005) 476-484.
- [17] M. Shikanai, K. Nakajima, T. Kawauchi, N-cadherin regulates radial glial fiber-dependent migration of cortical locomoting neurons, *Commun Integr Biol* 4 (2011) 326-330.
- [18] T.M. Kim, W. Huang, R. Park, P.J. Park, M.D. Johnson, A developmental taxonomy of glioblastoma defined and maintained by MicroRNAs, *Cancer Res* 71 (2011) 3387-3399.
- [19] Y. Liang, M. Diehn, N. Watson, A.W. Bollen, K.D. Aldape, M.K. Nicholas, K.R. Lamborn, M.S. Berger, D. Botstein, P.O. Brown, M.A. Israel, Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme, *Proc Natl Acad Sci U S A* 102 (2005) 5814-5819.

- [20] R. Mita, J.E. Coles, D.D. Glubrecht, R. Sung, X. Sun, R. Godbout, B-FABP-expressing radial glial cells: the malignant glioma cell of origin?, *Neoplasia* 9 (2007) 734-744.
- [21] U.E. Gasser, M.E. Hatten, Central nervous system neurons migrate on astroglial fibers from heterotypic brain regions in vitro, *Proc Natl Acad Sci U S A* 87 (1990) 4543-4547.
- [22] M.F. Santiago, S.S. Liour, R. Mendez-Otero, R.K. Yu, Glial-guided neuronal migration in P19 embryonal carcinoma stem cell aggregates, *J Neurosci Res* 81 (2005) 9-20.
- [23] M. Maden, L.A. Manwell, B.K. Ormerod, Proliferation zones in the axolotl brain and regeneration of the telencephalon, *Neural Dev* 8 (2013) 1.
- [24] V.S. Mashanov, O.R. Zueva, J.E. Garcia-Arraras, Radial glial cells play a key role in echinoderm neural regeneration, *BMC Biol* 11 (2013) 49.
- [25] M.M. Romero-Aleman, M. Monzon-Mayor, C. Yanes, D. Lang, Radial glial cells, proliferating periventricular cells, and microglia might contribute to successful structural repair in the cerebral cortex of the lizard *Gallotia galloti*, *Exp Neurol* 188 (2004) 74-85.
- [26] R. Nat, M. Nilbratt, S. Narkilahti, B. Winblad, O. Hovatta, A. Nordberg, Neurogenic neuroepithelial and radial glial cells generated from six human embryonic stem cell lines in serum-free suspension and adherent cultures, *Glia* 55 (2007) 385-399.

[27] S.S. Liour, R.K. Yu, Differentiation of radial glia-like cells from embryonic stem cells, *Glia*

42 (2003) 109-117.

FIGURE LEGENDS

Figure 1. Generation of fluorescently labeled cells and molecular characterization of GDC90 cells.

A: Construction of the PiggyBac transposon fluorescent protein (FP) expression vector. The CAG promoter enables strong expression in NPCs, and fluorescently labeled cells can be selected by neomycin resistance.

B-K: Proliferating GDC90 aggregates, other glial cell lines, and iPSC-NPC neurospheres with stable and strong fluorescent protein expression. Scale bar: 200 μ m

L-P: Immunocytochemistry of GDC90. GDC90 cells expressed the neural stem/progenitor cell markers Nestin and SOX2 and the RG cell markers GFAP and BLBP. Scale bar: 50 μ m

J-L: Quantitative RT-PCR analysis of GDC90. GDC90 cells expressed Nestin and GFAP and higher levels of BLBP than U-251MG cells (**P<0.001, *P<0.01).

Figure 2 Fluorescence imaging of co-cultured iPSC-NPCs and GDC90 cells.

A: Schematic of the adjacent co-culture system. iPSC-NPC neurospheres and glial aggregates were plated in contact on the same dish. Glial cells and iPSC-NPCs were distinguished by their

expression of tdTomato and EGFP, respectively.

B, C: Numerous iPSC-NPC-derived neurites were observed extending along GDC90 fibers, toward GDC90 aggregates. Scale bars: 500 and 200 μm in B and C, respectively

D, E: The other glioma cell lines, U-251MG and U-87MG, did not form polarized fibers and attracted fewer neurites. Scale bar: 200 μm

F: Schematic of mixed co-culture system. Aggregates containing both glial cells and iPSC-NPCs were plated.

G, H: GDC90-derived polarized radial fibers spread from the mixed aggregates. iPSC-NPCs-derived neurites were spread along GDC90 radiating fibers. The neurites ran efferently from the GDC90 cells. Scale bar: 200 μm

I, J: Cells of the other glioma-derived cell line GDC40 were not polarized and showed random fiber spreading. The co-cultured iPSC-NPCs did not become polarized and did not exhibit regular neurite extension.

K-L: Immunocytochemistry of a neuronal marker in iPSC-NPCs. In both the adjacent and mixed co-cultures, β III tubulin-positive (blue) neurites extended radially and exhibited EGFP fluorescence. The iPSC-NPC-derived neuronal cell bodies (arrow) were migrating away from

the aggregates. Scale bar: 50 μ m

M-N: Immunocytochemistry of DCX, a marker of migrating young neurons. A migrating EGFP-positive iPSC-NPC-derived neuron expressed DCX in its leading process (arrowhead).

Another DCX-positive neuron labeled with EGFP fluorescence was observed migrating along a GDC90 radial fiber that was identified by its tdTomato fluorescence (arrow). Scale bar: 50 μ m

Figure 3. GDC90 cells exhibit a higher affinity for iPSC-NPCs than do other glioma-derived cells

A-C: Fluorescence imaging of iPSC-NPCs and glioma-derived cells just after forming mixed aggregates in V-bottom plates.

D-I: Glioma-derived cells exhibited different affinities for iPSC-NPCs. On day 1 (D-F), iPSC-NPCs formed patchy aggregates with U-251 or U-87MG cells. By day 6 the iPSC-NPCs were excluded from the U-251 or U-87MB aggregates, while iPSC-NPCs remained in the mixed aggregates with GDC90 cells, suggesting that GDC90 cells have a higher affinity for iPSC-NPCs than do other established glioma cell lines. Scale bar: 1 mm

J: Quantitative RT-PCR analysis of N-cadherin. N-cadherin expression levels were normalized

to that of the U-87MG glioma cell line. N-cadherin expression in GDC90 cells was at least twice that in other glioma cells lines (**P<0.001). iPSC-NPCs expressed high levels of N-cadherin, similar to that of the human neural stem cell line (hNSC).

Figure 4. Behavior of iPSC-NPCs co-cultured with GDC90 cells on a three-dimensional scaffold.

A-C: GDC90 cells directed iPSC-NPCs-derived neurite extension on a silicate fiber membrane.

When GDC90 and iPSC-NPCs mixed aggregates were plated on the artificial membrane, iPSC-NPC neurites extended along the spreading GDC90 fibers. Scale bar: 100 μm

D-F: Long-term co-culture of iPSC-NPCs and GDC90 cells on the silicate membrane.

iPSC-NPCs became polarized and extended long neurites along the membrane fibers. Scale bar: 500 μm and 200 μm , respectively in E and F

Adjacent: **GDC90**+iPSC-NPCs

Adjacent: **U-251MG**+iPSC-NPCs

Adjacent : **U-87MG**+iPSC-NPCs

Adjacent: **GDC90**+iPSC-NPCs

Mixed: **GDC90**+iPSC-NPCs

Mixed: **GDC40**+iPSC-NPCs

Mixed : **GDC90**+iPSC-NPCs