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TECHNICAL REPORT

Establishment of a Gorlin syndrome model from induced neural progenitor cells exhibiting constitutive GLI1 expression and high sensitivity to inhibition by smoothened (SMO)

Hajime Ikehara[1,2] · Katsunori Fujii[2] · Toshiyuki Miyashita[3] · Yu Ikemoto[1,3] · Marina Nagamine[1,3] · Naoki Shimojo[2] · Akihiro Umezawa[1]

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Abstract

The hedgehog signaling pathway is a vital factor for embryonic development and stem cell maintenance. Dysregulation of its function results in tumor initiation and progression. The aim of this research was to establish a disease model of hedgehog-related tumorigenesis with Gorlin syndrome-derived induced pluripotent stem cells (GS-iPSCs). Induced neural progenitor cells from GS-iPSCs (GS-NPCs) show constitutive high GLI1 expression and higher sensitivity to smoothened (SMO) inhibition compared with wild-type induced neural progenitor cells (WT-NPCs). The differentiation process from iPSCs to NPCs may have similarity in gene expression to Hedgehog signal-related carcinogenesis. Therefore, GS-NPCs may be useful for screening compounds to find effective drugs to control Hedgehog signaling activity.

Keywords induced pluripotent stem cells · hedgehog signaling pathway · PTCH1 · GLI1 · vismodegib

Introduction

The hedgehog (Hh) signaling pathway plays an important role in embryogenesis and stem cell maintenance [1–3]. Ectopic Hh signal upregulation causes tumors such as basal cell carcinoma (BCC) and medulloblastoma [4, 5]. Hh signaling is also related to other malignancies including breast cancer, pancreatic cancer, lung cancer, and prostate cancer [6]. Many medications have been studied to establish treatment and the prevention of Hh signaling pathway-related tumors [7]. The Hh signaling pathway can be initiated by three ligands: desert hedgehog, Indian hedgehog, or sonic hedgehog [8]. These proteins bind to the 12-pass transmembrane protein receptor PTCH1. Once ligand binding occurs, the PTCH1 receptor relieves its inhibitory action on smoothened (SMO), a 7-pass transmembrane G-protein-coupled signal transduction molecule, which then activates a signaling cascade resulting in the translocation of Gli transcription factors to the nucleus. In the absence of the ligand, SMO is normally localized in vesicles. When the pathway is activated, SMO localizes to the primary cilium on the cell membrane.

Gorlin syndrome (GS) or nevoid basal cell carcinoma syndrome (NBCCS; OMIM 109400) is a rare autosomal dominantly inherited disorder that is characterized by congenital anomalies and development of tumors such as BCC and medulloblastoma [9, 10]. GS is caused by mutations in the PTCH1 gene and is transmitted as an autosomal dominant trait with complete penetrance and variable expressivity. Haploinsufficiency of PTCH1 results in constitutive activation of the Hh signaling pathway. Multiple genes that influence embryogenesis and pluripotency are oncogenes that drive the development of tumors [11–13] and some mechanisms of tumorigenesis are similar to embryonic development [14–17]. GS patients frequently develop medulloblastoma at a young age compared with patients with sporadic cases due to the mutation of PTCH1 which causes dysregulation of the Hh signaling pathway during development of the cerebellum [18].

✉ Akihiro Umezawa
umezawa@1985.jukuin.keio.ac.jp

[1] Center for Regenerative Medicine, National Center for Child Health and Development Research Institute, 2-10-1, Okura, Setagaya, Tokyo 157-8535, Japan

[2] Department of Pediatrics, Chiba University Graduate School of Medicine, Chiba, Japan

[3] Department of Molecular Genetics, Kitasato University School of Medicine, Kanagawa, Japan

Table 1 Patient information and PTCH1 gene mutations

Patients	Type of mutation	Nucleotide change	Amino acid change	Phenotype (Major criteria)
G11	Frameshift	c.3130_3131dupGC	p.V1045LfsX23	2, 3, 4, 5, 6
G12	Frameshift	c.3130_3131dupGC	p.V1045LfsX23	2, 3, 4, 6
G36	Deletion of the whole *PTCH1* gene			1, 2, 3, 4, 5
G72	Frameshift	c.274delT	p.C92VfsX25	2, 3, 4, 5

Major criteria include 1: more than two basal cell carcinomas or one under the age of 20 years, 2: odontogenic keratocysts of the jaw proven by histology, 3: three or more palmar or plantar pits, 4: bilamellar calcification of the falx cerebri, 5: bifid, fused, or markedly splayed ribs, 6: first degree relative with NBCCS

Ptch1$^{+/-}$ mice, an animal model of GS, develops medulloblastoma in early life stages [19, 20], and develops BCC-like tumors in their skin from exposure ultraviolet light or radiation exposure [21]. There are few reports, however, of tumorigenesis models with human cells. Differences between human and other vertebrate Hh signaling pathways may make it difficult to precisely predict effects and side effects of a candidate drug to treat Hh pathway-related tumors [22]. Disease models using human induced pluripotent stem cells (iPSCs) have recently been described as a promising tool for the investigation of disease mechanisms and identification of new drugs because of their pluripotency [23–26]. iPSCs are capable of differentiating into many types of cells in vitro, including neural cells [27–30]. Hence, neural cells from iPSCs can be a valuable resource to investigate the efficacy and safety of drugs, including Hh signaling inhibitors. Vismodegib (CDC-044), inhibitor of SMO, is the first oral medicine to be approved by the US Food and Drug Administration that disrupts the Hh pathway for treatment of advanced phase BCC in adults [31]. However due to its many side effects, most of patients do not continue treatment [32]. Safer and more tolerable drugs are necessary.

In this study, we generated GS-iPSCs with a heterozygous mutation of PTCH1, and induced neural progenitor cells (NPCs) from GS-iPSCs. Neural progenitors from GS-iPSCs expressed GLI1, a downstream target gene of Hh signaling, and exhibited sensitivity to Vismodegib, an SMO inhibitor. Induced NPCs from GS-iPSCs may therefore serve as a good precancerous model for Hh-related tumors.

Materials and methods

iPSC generation from GS patients

In an earlier study, Ikemoto et al. established iPSCs from four different GS patient fibroblasts by Sendai virus infection of four genes (OCT3/4, SOX2, c-MYC, and KLF4) [33, 34]. The four GS patients' genetic mutations are listed in Table 1. Human iPSCs derived from fetal lung fibroblasts (MRC5) and menstrual blood cells (Edom) that were previously established in our laboratory were used as wild-type control iPSCs (WT-iPSCs) [35].

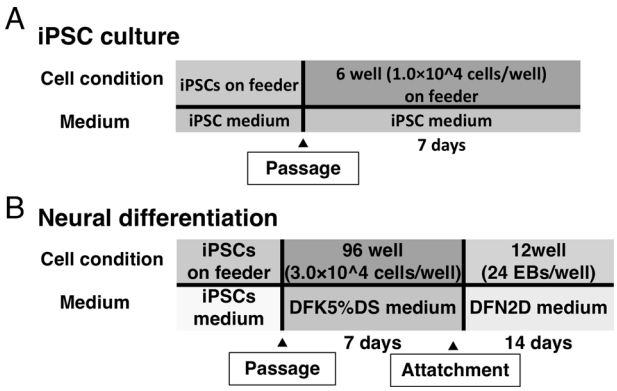

Fig. 1 iPSC culture protocol and neural induction protocol (modified SFEBq). **a** iPSCs were maintained on irradiated mouse embryonic fibroblasts (MEF), and analyzed on the 7th day after the last passage. **b** NPCs were induced from the iPSCs with a modified SFEBq protocol, and analyzed on 21st day after formation of embryonic body

Cell culture

Undifferentiated human iPSCs were maintained on a feeder layer of irradiated mouse embryonic fibroblasts (MEF) on a gelatin-coated dish in a human iPSC medium at 37 °C in humidified air with 5% CO_2. The human iPS medium consisted of KnockOut Dulbecco's modified eagle medium (KO-DMEM), KnockOut serum replacement (KSR), GlutMAX, nonessential amino acids(NEAA), 2-mercaptoethanol, penicillin/streptomycin, sodium pyruvate, and bFGF (all from Gibco). The medium was changed daily. Human iPSC colonies composed of closely packed cells were split approximately every 5–7 days by incubation in a dissociation solution for 5 min at 37 °C and passaged onto new irradiated MEF.

Neural progenitor cell induction

iPSCs were dissociated to single cells and quickly reaggregated in U-bottom 96-well plates (Thermo Fisher) for suspension culture. Aggregations, or embryoid bodies (EB), were cultured in "DFK5% medium" (DFK5%; DMEM/Ham's F12 (Gibco, USA) supplemented with 5% KSR (Gibco), NEAA (Gibco), 0.1 M 2-mercaptoethanol (Gibco))

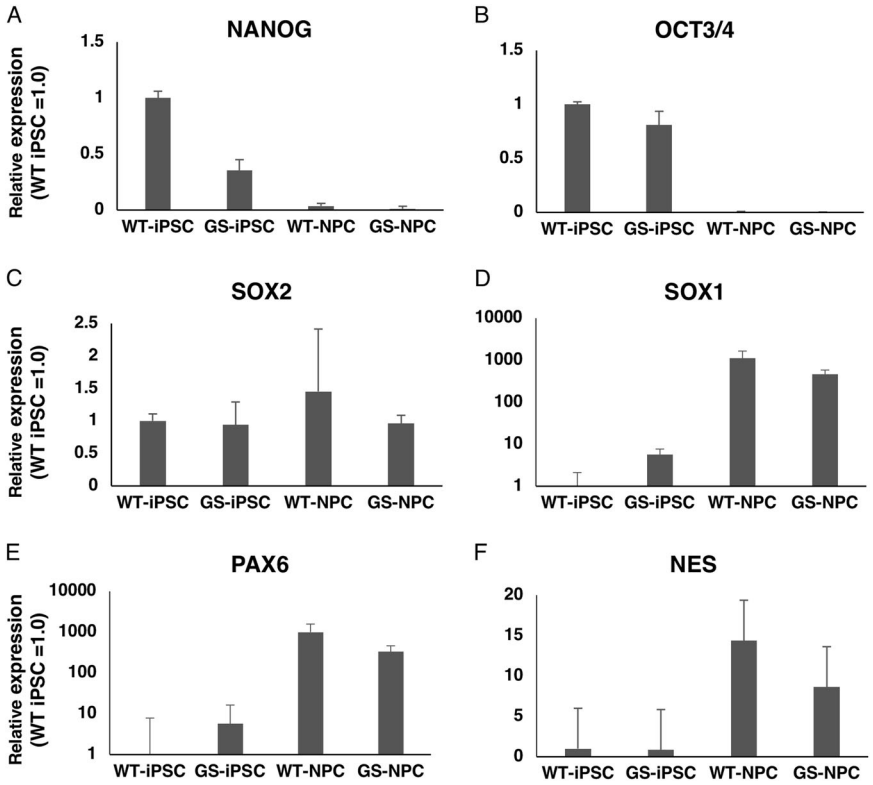

Fig. 2 Expression levels of marker genes for pluripotent and neural cells. **a–f** The expression levels of NANOG, OCT3/4, SOX2, SOX1, PAX6, and NES in undifferentiated human iPSCs and induced neural progenitor cells (NPCs). Each bar represents the mean ± SEM from two WT-iPSCs ($n = 2$) and four GS-iPSCs ($n = 4$). The graphs represent the relative gene expression levels when the level in WT-iPSC is defined as 1.0

with 2 μM dorsomorphin (Wako) and 10 μM SB431542 (Wako) in a neural inductive stage (day 0–7). After neural induction, EB were transferred onto Matrigel (Corning)-coated 12-well culture plates and cultured in "DFN2D medium" (DMEM/Ham's F12 (Gibco, USA) supplemented with 1× N_2 supplement (Invitrogen), NEAA (Thermo Fisher), 0.1 M 2-mercaptoethanol (Gibco)) with 2 μM dorsomorphin (Wako) day 7–21).

SMO inhibitor (Vismodegib), GLI1 inhibitor (GANT61), and SMO agonist (SAG) treatment

The cells were treated with 10 μM Vismodegib (Selleckchem), or 10 μM GANT61 (SANTA CRUZ), or 5 μM SAG (Wako) for 48 h after differentiation protocol (Fig. 4). The compounds were dissolved in DMSO, which was added to the culture medium at a final concentration of 0.1%.

Quantitative real-time PCR

RNA was extracted from cells using the RNeasy Micro kit (Qiagen). An aliquot of total RNA was reverse transcribed using an oligo (dT) primer. For the thermal cycle reactions, the cDNA template was amplified with gene-specific primer sets using the Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen) under the following reaction conditions: 40 cycles of PCR (95 °C for 15 s and 60 °C for 1 min) after an initial denaturation (95°C for 2 min). Fluorescence was monitored during every PCR cycle at the annealing step. The authenticity and size of the PCR products were confirmed using a melting curve analysis (using software provided by Applied Biosystems). mRNA levels were normalized using GAPDH as a housekeeping gene.

Statistical analysis

Data are represented as mean ± standard error of the mean and were compared using a Welch's t test. A p value < 0.05 was considered significant.

Results

iPSC culture and neural differentiation protocol

The Hh signaling pathway is an important factor in embryogenesis and stem cell maintenance, and its dysregulation leads to tumorigenesis. The difference between WT and GS-induced neural cell characteristics would be a key point of disease model. We employed a modified SFEBq protocol as an efficient differentiation method of iPSCs [36] and successfully induced iPSCs to NPCs (Fig. 1). GS-iPSCs from four GS patients were used for this study (Table 1). No obvious genotype-clinical phenotype relationship was detected. GS- and WT-iPSCs exhibited

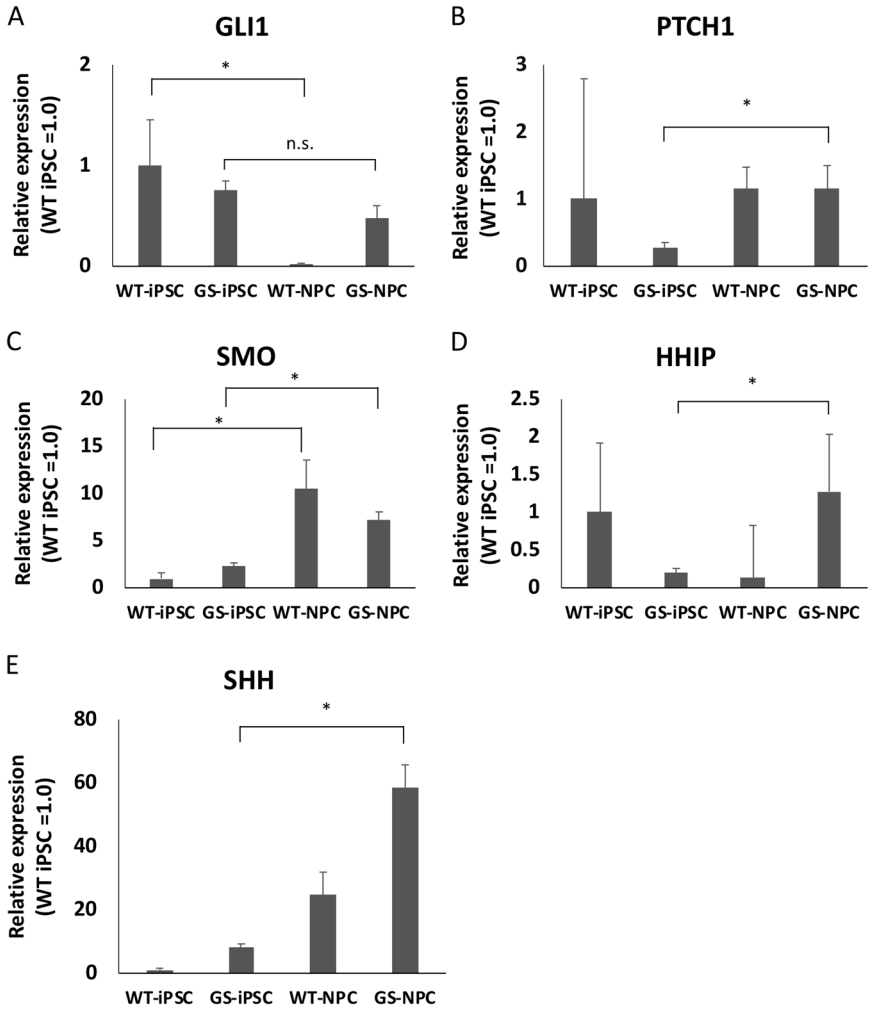

Fig. 3 Expression level of Hh signaling pathway-related mRNA. The expression level of Hh signaling pathway-related genes (**a** GLI1, **b** PTCH1, **c** SMO, **d** HHIP, **e** SHH) in undifferentiated human iPSCs and NPCs. Each bar represents the mean ± SE from two WT-iPSCs ($n = 2$) and four GS-iPSCs ($n = 4$). The graph represents the gene expression levels of GS-iPSC, WT-NPC, and GS-NPC, relative to that of WT-iPSC (defined as 1.0). The asterisk indicates a significant difference ($p < 0.05$). n.s. no statistical significance

similar cell morphology and growth in vitro. GS- and WT-iPSCs expressed pluripotency markers, i.e. NANOG, OCT3/4, and SOX2 (Fig. 2). NANOG and OCT3/4 expression levels decreased to undetectable levels with neural differentiation. SOX2, a marker for both neural stem cells and neural progenitors, was expressed at the same level in both iPSCs and NPCs. Neural progenitor makers, i.e. SOX1, PAX6, and Nestin, were upregulated in WT-NPCs and GS-NPCs.

Expression of Hedgehog signal-related genes in iPSCs and NPCs

The expression level of GLI1 in WT-iPSCs decreased remarkably after neural differentiation [37, 38], whereas the GS-NPCs maintained their GLI1 expression level (Fig. 3a). The expression of two other Hh signaling pathway target genes, PTCH1 and Hedgehog interacting protein (HHIP), increased in GS-NPCs (Fig. 3b, d). Expression levels of SMO (the main activator of Hh signaling pathway) and SHH (a ligand of Hh) increased in WT-NPCs and GS-NPCs when compared with WT- and GS-iPSCs, respectively (Fig. 3c, e). GS-NPCs maintained high GLI1 transcriptional levels and showed higher Hh signal activity due to the heterozygous mutation of PTCH1, which is the major Hh signal pathway repressor.

Effects of SMO inhibitor (Vismodegib), GLI inhibitor (GANT61), and SMO agonist (SAG)

To investigate the effect of SMO inhibition, Gli inhibition, and SMO agonism, iPSCs and NPCs were exposed to Vismodegib, GANT61, and SAG for 48 h (Fig. 4a). The expression levels of Hh target genes (GLI1, HHIP, and PTCH1) were compared with mock cells (treated only with drug solvent) after the drug stimulation (Fig. 4b). The high expression levels of GLI1 in GS NPCs were drastically suppressed by Vismodegib, most likely due to their higher sensitivity to SMO inhibition. The reduction in the expression of HHIP was higher than that of protein patched homolog 1 (PTCH1) in GS-NPCs (Fig. 4c, d). GANT61, another promising small molecule that suppresses GLI

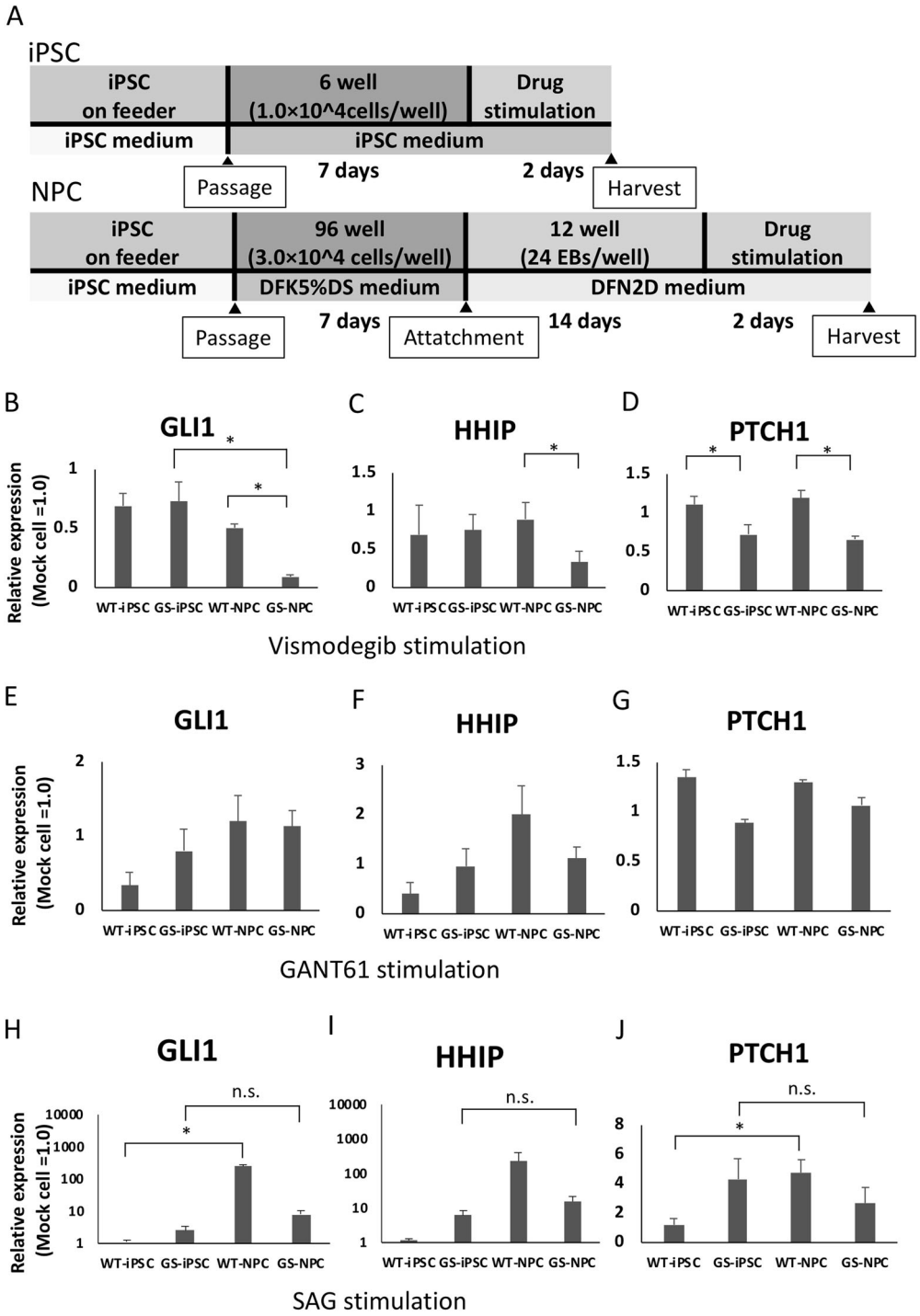

Fig. 4 Effects of SMO inhibitor, GLI inhibitor, and SMO agonist on expression of Hh signal target genes. **a** iPSCs and NPCs were treated with SMO inhibitor (Vismodgegib), GLI inhibitor (GANT61), and SMO agonist (SAG) for 48 h. **b–j** mRNA expression of hedgehog target genes (GLI1, HHIP, and PTCH1) was analyzed using quantitative RT-PCR. The graphs represent the expression of GS-iPSC, WT-NPC, and GS-NPC relative to that of the control cells (WT-iPSC). Each bar represents mean ± SE from WT-iPSCs ($n = 2$) and GS-iPSCs ($n = 4$). The asterisk indicates a significant difference ($p < 0.05$). n.s. no statistical significance

expression [39], failed to inhibit GLI1 expression in GS-iPSCs and GS-NPCs (Fig. 4e–g). Hh target genes were highly expressed in GS-NPSs and drug sensitivity to SAG was unchanged (Fig. 4h–j).

Discussion

In this study, we introduced a screening system to find effective drugs to control Hh signaling activity and a good

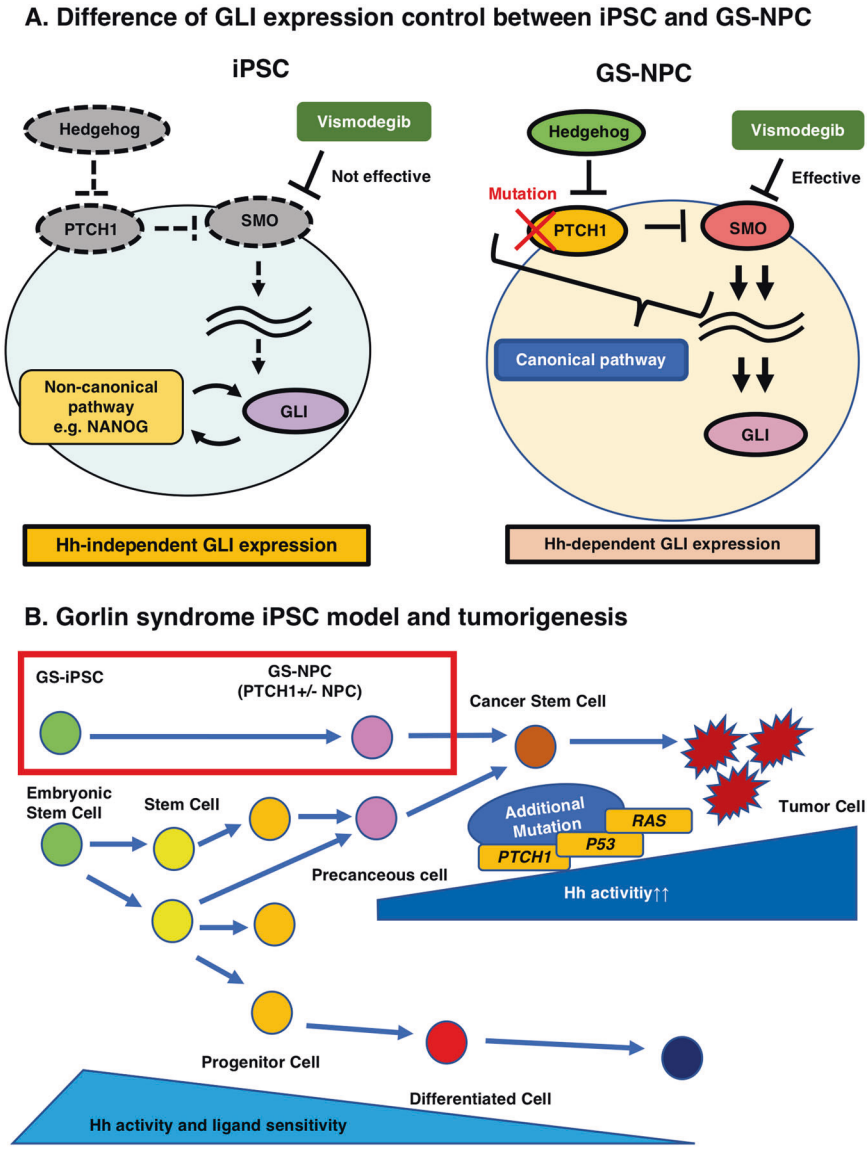

Fig. 5 Gorlin syndrome iPSC model. **a** Difference of GLI expression control between iPSC and GS-NPC. In iPSCs, Hh-PTCH1-SMO-GLI signaling axis (canonical pathway) is silenced. GLI interacts with another noncanonical pathway such as NANOG. Therefore, Vismodegib is not effective. In GS-NPCs, the canonical pathway is activated due to neural differentiation and heterozygous mutation of PTCH1. Vismodegib is effective in GS-NPCs. **b** Gorlin syndrome iPSC model and tumorigenesis. In iPSCs, the Hh signaling pathway is silent. Hh signaling pathway is activated with differentiation, and plays important roles in stem cell maintenance and differentiation. The Hh signaling pathway is strictly controlled in WT cell and decreases with terminal differentiation. Mutation of PTCH1 causes uncontrolled Hh signaling activation (precancerous cells), and additional mutation of oncogenes such as RAS and P53 results in development of cancer stem cells

precancerous model for Hh-related tumors. GS-NPCs can be used as a drug screening system, but GS-iPSCs may not be available because of the lack of Hh signaling. Hh target genes such as GLI1, HHP, and PTCH1 in GS-NPCs are considered to be promising biomarkers to assess efficacy of drugs that control Hh signaling pathway. With these biomarkers, it may be possible to perform drug screening to find a novel compounds that moderate SMO activity.

iPSC-based screening system for GS

Vismodegib was effective in GS-NPC, but not very effective in GS- and WT-iPSCs. Differential Vismodegib response in the iPSC and NPC is possibly explained by transcriptional regulation of the GLI gene (Fig. 5a). In undifferentiated iPSCs, GLI interacts with another noncanonical pathway such as NANOG in iPSCs [38, 40, 41]. The SHH-PTCH1-SMO-GLI1 signaling axis (the canonical Hh pathway) was indeed silenced in both GS- and WT-iPSCs, and activated with differentiation. Further activation of SMO and its downstream pathway is attributed to heterozygous mutation of PTCH1 in the GS-NPCs. The marked inhibition seen with the SMO inhibitor in GS-NPCs also supports the usefulness of GS-NPCs for drug screening. It is also noted that SHH-PTCH1-SMO-GLI1 signaling is present in NPCs as shown by signal activation with the SMO activator despite the of lack of GLI1 expression in WT-NPCs. Low basal expression level of GLI1 in WT-NPCs was much lower than iPSCs, but was significantly upregulated by the SMO agonist. This is probably due to increased Hh signaling caused by neural differentiation. Further studies regarding the correction of PTCH1 gene mutation in GS-NPCs using gene

editing techniques may elucidate the precise role of the PTCH1 gene in modifying drug sensitivity to SMO antagonist.

The neural differentiation protocol employed in this study may produce a heterogenous population; in addition to terminally differentiated neurons, glia, and oligodendrocytes, immature neural cells and neural stem cells may be present. This diverse cell population will be an effective drug screening system since the biomarker GLI for screening is activated only in differentiated cells and the increased GLI expression is inhibited by the SMO inhibitor. GS-NPCs are therefore suitable cell for drug screening. In mice, SMO is dispensable to cell survival in very early embryogenesis [42]. $SMO^{-/-}$ mutant embryonic cells are able to develop to the morula stage and survive in a mosaic embryo when mixed with SMO wild-type cells. At the start of embryogenesis, Hh signaling is activated and SMO becomes necessary for signal transduction [43]. SMO mutant embryos fail to differentiate neural cells. Likewise, SHH-PTCH1-SMO-GLI1 signaling may be dispensable in survival and proliferation of iPSCs that have phenotypes of embryonic stem cells and an inner cell mass of blastocysts.

GS-NPC and Hh-related tumorigenesis

High GLI1 expression in GS-NPCs is compatible with an uncontrolled Hh signal negative feedback loop due to the PTCH1 mutation. The extremely high expression of GLI1 possibly causes GS-NPCs to be vulnerable to carcinogens and causes us to postulate that GS is a cancer-susceptibility disorder. High GLI1 levels are directly related to tumorigenesis and further oncogene mutation initiation [44, 45]. Overexpression of GLI1 can cause malignancy in the skin of mice [46–48]. Therefore, controlling GLI1 expression is important for the prevention of tumorigenesis. GS-NPCs are a useful disease model of precancerous cells from the viewpoint of GLI1 transcriptional regulation (Fig. 5b). In embryogenesis, the Hh signaling pathway is transiently activated, resulting in differentiated or progenitor cells. With mutation of an oncogene, stem cells and progenitor cells transform to precancerous cells, and an additional oncogene mutation results in cancer stem cells. GS-NPCs are available as equivalent to precancerous cells and good disease model of Hh-related tumorigenesis. These results stress the need for control of GLI1 expression to prevent tumor formation and tumor progression in GS patients and Hh signaling pathway-related tumors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement This study was approved by Ethics committee of Chiba University Graduate School of Medicine (#2017 - 792), and the Institutional Review Board of the National Center for Child Health and Development of Japan (#884). We obtained informed consent from each patient with Gorlin syndrome. Animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the National Research Institute for Child Health and Development (A2003-002-C15-M06).

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