千葉大学学位申請論文

# Hedgehog signaling is synergistically enhanced by nutritional deprivation and ligands stimulation in human fibroblasts of Gorlin syndrome

（ゴーリン症候群ヒト線維芽細胞におけるヘッジホッグシグナルは栄養飢餓とリガンド刺激により相乗的に亢進する）

千葉大学大学院医学薬学府

先進医療科学専攻

（主任：下条直樹教授）

水落　弘美

## Abstract

Hedgehog signaling is a pivotal developmental pathway that comprises hedgehog, PTCH1, SMO, and GLI proteins. Mutations in *PTCH1* are responsible for Gorlin syndrome, which is characterized by developmental defects and tumorigenicity. Although the hedgehog pathway has been investigated extensively in *Drosophila* and mice, its functional roles have not yet been determined in human cells. In order to elucidate the mechanism by which transduction of the hedgehog signal is regulated in human tissues, we employed human fibroblasts derived from three Gorlin syndrome patients and normal controls. We investigated GLI1 transcription, downstream of hedgehog signaling, to assess native signal transduction, and then treated fibroblasts with a recombinant human hedgehog protein with or without serum deprivation. We also examined the transcriptional levels of hedgehog-related genes under these conditions. The expression of GLI1 mRNA was significantly higher in Gorlin syndrome-derived fibroblasts than in control cells. Hedgehog stimulation and nutritional deprivation synergistically enhanced GLI1 transcription levels, and this was blocked more efficiently by vismodegib, a SMO inhibitor, than by the natural compound, cyclopamine. Messenger RNA profiling revealed the increased expression of Wnt signaling and morphogenetic molecules in these fibroblasts. These results indicated that

the hedgehog stimulation and nutritional deprivation synergistically activated the hedgehog signaling pathway in Gorlin syndrome fibroblasts, and this was associated with increments in the transcription levels of hedgehog-related genes such as those involved in Wnt signaling. These fibroblasts may become a significant tool for predicting the efficacies of hedgehog molecular-targeted therapies such as vismodegib.

## 1. Introduction

Hedgehog signaling is a pivotal developmental pathway that comprises hedgehog, patched, smoothened, and gli proteins [1]. The hedgehog gene was first identified in *Drosophila* as a segment polarity gene that controls the segmental pattern of the embryo [2]. Hedgehog signaling is known to be crucially involved in morphogenesis, tumorigenesis, osteoblast differentiation, wound repair, and neuroprotection [3,4].

PTCH, the human homolog of *Drosophila* patched, is a twelve-pass transmembrane protein coding a receptor for hedgehog that constitutively suppresses activation of the hedgehog pathway by inhibiting SMO, which transmits hedgehog signaling. When hedgehog ligands bind to PTCH, the inhibition of SMO is released and SMO then activates GLI through several cytoplasmic transduction steps, leading to the translocation of GLI into the nucleus and expression of the targeted gene [5].

Gorlin syndrome (GS) is a rare autosomal dominant disease that is characterized by developmental anomalies including palmer and plantar pits, skeletal abnormalities, falx calcification, and tumorigenesis such as basal cell carcinoma (BCC), ketatocystic odontogenic tumors, and medulloblastoma [6]. This syndrome is caused by mutations in *PTCH1*, and more than 200 mutations have been reported to date [7].

Tumorigenesis is a lifelong problem in patients with GS. These patients develop

BCCs in their early 20s, with more than 80% of patients having one or more BCCs throughout their lifetime. Medulloblastoma occurs in 4% of patients with GS at a mean age of 2.3 years [8]. Molecular targeted inhibitors of the hedgehog pathway have recently been developed to treat these tumors. Several small compounds have been applied to clinical trials for these tumors [9,10,11]. Of these, vismodegib (GDC-0449) was found to be the most effective inhibitor of SMO, reducing the number of BCCs [9].

Since the hedgehog pathway has not yet been elucidated in detail in human cells, we herein investigated this signaling pathway in GS-derived dermal fibroblasts under diverse cellular stress conditions.

## 2. Material and Methods

### *2.1. Materials*

Recombinant human sonic hedgehog (shh) (C24II), N-Terminus (R&D Systems, Minneapolis, USA), smoothened agonist (SAG) (Calbiochem, Darmstadt, Germany), cyclopamine (BIOMOL, PA, USA), and vismodegib (GDC-0449) (Selleckchem, Huston, TX, USA) were purchased. Mouse monoclonal antibodies against human acetylated tubulin (Sigma-Aldrich, Saint Louis, MO, USA), anti-mouse IgG Alexa 594 (Abcam, Cambridge, UK), and ‘4’,6-diamidini-2-phenylindole, dihydrochloride (DAPI) (Roche Diagnostics, Mannheim, Germany) were obtained.

### *2.2. Human dermal fibroblasts*

All studies described below were approved by the local Ethics Committee of Chiba University. Human dermal fibroblasts were collected from three Japanese GS patients, who were from two families with *PTCH1* mutations as an insertion in one family (G11 and G12; c.3130_3131dupGC) and a deletion in one patient (G72 c.272delG). The ages of G11, G12, and G72 at dermal fibroblast collection were 14, 42, and 36 years old, respectively. After obtaining informed consent from GS patients, dermal specimens were collected from unaffected skin areas at the time of surgical operations. These

surgically resected dermal tissues were then cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin, and 1% streptomycin. Fibroblasts were obtained from these dermal tissues, maintained at 37℃ in a humidified atmosphere (5% $CO_2$ and 95% air), and seeded on 10-mm dishes. The culture medium was renewed every 2 days until cellular confluence was achieved. Three origin-different normal human adult dermal fibroblasts were purchased (PCS-201-012; ATCC, Manassas, VA, USA). These fibroblasts were used during passages 4 to 8 in subsequent experiments.

*2.3. Stimulation with agonists and antagonists of the hedgehog pathway*

Fibroblasts were seeded on 12-well plates in DMEM with 10% FBS for 24hrs, the culture medium was renewed before stimulation experiments, and DMEM with 0.5% FBS was used for serum starvation. These fibroblasts were treated with human recombinant shh (1000ng/ml), cyclopamine (100nM), SAG (1μM), and vismodegib (10nM) for 48hrs after the medium change.

*2.4. Immunofluorescence and confocal microscopy*

Human fibroblasts grown on slide chambers were fixed with 4% paraformaldehyde.

Cells were washed with phosphate buffer saline (PBS) and permeabilized with 0.2% Triton X-100 in PBS, followed by blocking with 2% bovine serum albumin (BSA) in PBS for 1 hr at room temperature. Cells were incubated overnight at 4℃ with primary antibodies, diluted in 2% BSA in PBS, as mouse monoclonal anti-acetylated α-tubulin (1:1000). After washing with PBS, cells were incubated with secondary antibodies, anti-mouse IgG Alexa 594 (1:500), and DAPI (1:1000) for 30 min. Cells were viewed under a FLUOIVIEW FV1000-D/FV10i confocal microscope (OLYMPAS, Tokyo, Japan) and counted in at least 10 views.

*2.4. mRNA quantification by real-time PCR*

RNA was extracted from human dermal fibroblasts using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized with SuperScript Ⅲ First Strand Synthesis SuperMix (Invitrogen). PCR primers were designed against GLI1, GLI2, GLI3, PTCH1b, and SMO which are described in Table 1, and the reagent mix was incubated on the cycler (Bio-Rad, CA, USA) with normalization by the internal control gene human GAPDH. PCR reactions were run for 40 cycles twice in triplicate. The log-linear phase of amplification was monitored to obtain threshold cycle values. The comparative threshold cycle method was applied to determine expression levels.

The absence of primer dimers was verified by running the PCR product on a 1.5% agarose gel. To explore the relationships among hedgehog pathway components, we employed the RT2 Profiler PCR Array (Qiagen) and analyzed them by RT2 Profiler PCR Data Array analysis v3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

*2.5. Statistical analysis*

Data were compared by a two-way ANOVA and Dunnett's test using PRISM6 (Graph Pad software). A *P* value less than 0.05 were considered significant. Data were shown as the mean ± the standard error of the mean (SEM).

## 3. Results

*3.1. Hedgehog signaling was enhanced in GS fibroblasts by shh with serum deprivation.*

To establish the untreated status of hedgehog signaling in GS fibroblasts, we measured the mRNA levels of components of the hedgehog pathway: GLI1, GLI2, GLI3, PTCH1b, and SMO, using real-time PCR. Before the treatment with shh, GLI1 mRNA levels were more than 3-fold higher in GS fibroblasts than in the controls (Figure 1A). The expression levels of SMO mRNA were also 2-fold higher in GS fibroblasts than in the controls. No significant differences were observed in the mRNA levels of the other hedgehog pathway components between GS fibroblasts and the controls. Although GLI mRNA levels were elevated in all GS fibroblasts, individually different activated levels were observed in each GS cell. For example, GLI1 levels were elevated less in G12 fibroblasts than in other GS fibroblasts.

We then investigated the influence of shh on GS fibroblasts in order to determine whether this condition represented tumorigenesis in GS patients. The sole stimulation of the hedgehog protein did not enhance GLI1 mRNA levels above those in the controls (Figure 1B).

Since the number of ciliated cells has been shown to increase with nutritional deprivation [12] and hedgehog signaling passes through these primary cilia, we starved

fibroblasts and treated them with or without shh. The stimulation of shh and serum deprivation markedly elevated GLI1 levels by at least 3-fold those in the controls (Figure 1B). GLI1 mRNA levels were lower in G12 fibroblasts than in other GS fibroblasts under the untreated condition; however, these levels were significantly increased by the shh stimulation. These results suggested that GLI1 mRNA levels were constitutively higher in all GS fibroblasts than in the controls, and that hedgehog signaling was synergistically activated by shh stimulation and serum deprivation.

*3.2. Formation of primary cilia was increased after serum starvation*

We examined the formation of primary cilia by GS fibroblasts and controls using immunostaining for primary cilia and counted the number of ciliated cells (Figure 2A). The percentage of ciliated cells was increased by serum starvation in both GS fibroblasts and controls (Figure 2B). Under the condition of serum starvation, the additional shh stimulation did not increase the number of ciliated cells (Figure 2C). These results suggested that GS fibroblasts as well as control fibroblasts have the ability to form cilia under nutritional deprivation.

*3.3. Cyclopamine and vismodegib reduced shh-induced elevations in GLI1 levels in GS*

*fibroblasts*

To investigate the effects of hedgehog pathway activators and inhibitors on human fibroblasts, we used a SMO agonist (SAG) and SMO inhibitors, such as cyclopamine and vismodegib. We initially treated fibroblasts with SAG, which increased the transcription levels of GLI1 more than by the shh treatment alone (Figure 3A).

We next examined the effects of cyclopamine on GS fibroblasts. The shh-induced increase in Gli1 by shh was suppressed by cyclopamine to the levels observed under the untreated condition. On the other hand, no significant differences were observed in SMO mRNA levels between GS fibroblasts and the controls (Figure 3B).

Vismodegib is a SMO inhibitor that has been used to treat basal cell carcinoma and medulloblastoma in clinical settings. To investigate the effects of the inhibition caused by this reagent, we treated GS fibroblasts and control cells with vismodegib and measured GLI1 mRNA levels. In GS fibroblasts, vismodegib markedly reduced the shh-induced expression of GLI1 mRNA (Figure 3C).

Among the GLI1 levels observed, the reduction rates by cyclopamine and vismodegib were 44.5% and 53.7% in G11, 85.2% and 90.5% in G12, and 40.0% and 66.8% in G72, respectively (Figure 3A, 3C). These results suggested that vismodegib was a more effective anti-cancer reagent in human fibroblasts than cyclopamine. In

these experiments, GS fibroblasts responded to shh and SAG more effectively than control cells, and vismodegib clearly inhibited the hedgehog pathway in GS fibroblasts.

*3.4. Molecular survey for identification of hedgehog-related genes*

We detected different GLI1 mRNA levels between GS fibroblasts and normal controls in response to hedgehog pathway agonists and antagonists. To investigate the cause of these differences, we analyzed the expression levels of 84 hedgehog pathway-associated genes using a PCR array (Figure 4A).

Two-fold increases in expression levels between GS fibroblasts and control cells with both shh and serum deprivation were detected for GLI1, SMO, WNT8a (wingless 8a), WNT9b, ZIC1 (zinc finger protein of the cerebellum 1), and ZIC2 (Figure 4B). These results suggested that the WNT pathway and ZIC may interact with the hedgehog pathway in human fibroblasts. On the other hand, 2-fold decreases in expression levels between GS fibroblasts and control cells were observed for BMP4 (bone morphogenetic protein 4), BMP8A, FGF9 (fibroblast growth factor 9), SFRP1, WNT16, and WNT2b (Figure 4C). Among these six genes, the expression of BMP4 was decreased the most by the stimulation of GS fibroblasts with shh.

## 4. Discussion

We herein demonstrated that the GLI1 mRNA levels in dermal fibroblasts derived from patients with Gorlin syndrome were markedly increased. Hedgehog signaling was synergistically enhanced by hedgehog ligand binding and serum deprivation, and effectively blocked by the SMO inhibitor, vismodegib, which is already available on the market. These results revealed the distinctive usefulness of human dermal fibroblasts as a human specimen for examining hedgehog signal transduction, as well as mouse embryonic fibroblasts, and possibly evaluating the individual efficacies of molecular targeted therapies.

Human fibroblasts are evolutionarily conserved mesenchymal cells that potentially express hedgehog pathway proteins. Since hedgehog signaling is relatively conserved in mouse embryonic fibroblasts, biological responses to hedgehog ligands have been thoroughly investigated. However, experiments using human dermal fibroblasts have been limited [13,14], and recent developments in molecular targeted therapies have necessitated the use of a tool to establish whether a drug will be useful as an anti-cancer treatment. Therefore, we employed human dermal fibroblasts in the present study.

The most significant finding of this study was that hedgehog signaling in human fibroblasts was synergistically enhanced by hedgehog ligand binding and serum

deprivation. Given that hedgehog signaling is stimulated by the hedgehog protein, we treated human fibroblasts with the hedgehog protein under diverse conditions, resulting in the partial enhancement of the hedgehog pathway. However, the treatment of serum deprivation besides hedgehog binding markedly enhanced hedgehog signaling in GS fibroblasts. Thus, serum deprivation may potentially enhance hedgehog signaling in human tissues.

Primary cilia are a non-motile microtubule-based organelle that grows from the plasma membrane and functions in hedgehog signal transduction. In the absence of hedgehog, PTCH1 typically localizes to cilia and blocks the entry of SMO into primary cilia. However, in the presence of hedgehog, the inhibition by PTCH1 is released and SMO moves into primary cilia, resulting in signal activation [15,16,17]. Serum deprivation can induce the development of primary cilia, which has been associated with an increase in the number of ciliated cells [12]. Thus, we considered that the synergistic enhancement of hedgehog signaling may be transmitted via primary cilia. Since no significant difference was observed in the number of ciliated cells between GS fibroblasts and the controls, these enhancements in hedgehog signaling may have been due to novel mechanisms other than an increase in the number of ciliated cells.

Previous studies have examined the relationship between serum starvation and

cancer treatments. In human mesothelioma, lung carcinoma, and colorectal carcinoma cell lines, serum starvation enhanced the effects of cisplatin by activating ATM/Chk2/p53 [18]. Furthermore, when pediatric brain tumor cells were exposed to chemotherapeutics under starvation stress, sensitivity to chemotherapy was elevated [19]. Thus, nutritional deprivation may also contribute to the treatment of cancer and growth of cancer cells.

In our molecular survey for hedgehog-related genes, the expression of ZIC1 and ZIC2 were increased in the presence of both shh and serum deprivation. ZIC family proteins play an important role in neural development. In the early phase, in which the ectoderm differentiates into the neuroectoderm, ZIC controls the dorsal neural tube, while hedgehog controls the ventral one [20]. The dorsal expression of ZIC was shown to be regulated by sonic hedgehog as well as BMP4 and BMP7 [21]. The aberrant expression of ZIC1 and ZIC2 has been reported in some tumors, for example medulloblastoma and meningioma [22]. Interactions have also been detected between ZIC and GLI proteins through their zinc finger domains, suggesting that these interactions may depend on certain cell types [23, 24]. Thus, the results of our study suggested that ZIC and GLI systematically interacted and regulated cellular responses with each other in human fibroblasts.

In contrast, the expression of BMP4 was 10-fold lower in GS fibroblasts than in the controls in the presence of shh. BMP4, a member of the BMP family, plays important roles in embryogenesis and the development and maintenance of adult tissue homeostasis [25]. It has also been shown to inhibit the tumorigenicity of human glioblastoma cells *in vitro*, and may be a potential therapeutic agent [25,26]. These findings suggest that BMP4 is an alternative inhibitor of the hedgehog pathway and can be used in the treatment of hedgehog-related tumors.

There were limitations to this study. Different hedgehog activity levels were observed among the GS patients who participated in this study. We attributed this to the limitation of human primary fibroblasts. G12 fibroblasts had higher GLI1 mRNA levels and better responses to the Shh stimulation than the controls; however, these levels were lower than those of other GS fibroblasts. Given that human primary cells generally have a limited capacity for proliferation in cultures, this phenomenon may be related to reductions in telomere lengths [27]. Moreover, an inverse relationship has been reported between donor age and telomere length in dermal fibroblasts [28]. Since G12 was older than the two other patients, we could not exclude the possibility of a decrease in the viability of the G12 fibroblasts. In this study, we only examined GS fibroblasts from three patients; therefore, further investigations are needed to elucidate the mechanism

underlying these synergistically enhancements in human GS fibroblasts.

Our results suggest that hedgehog signaling is constantly activated in Gorlin syndrome fibroblasts. Given that this signaling is synergistically enhanced by ligand hedgehog binding and serum deprivation, inhibitors of the pathway with starvation may be applied as alternative therapeutic approaches for the treatment of Gorlin syndrome in the future.

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**Conflict of interest statement**

None declared.

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Table 1 Primers used for real-time PCR

| Probe | Forward primer (5′ to 3′)<br>Reverse primer (5′ to 3′) | Annealing temperature (°C) | Length (bp) |
|---|---|---|---|
| *GLI1* | AGGGAGTGCAGCCAATACAG<br>ATTGGCCGGAGTTGATGTAG | 64 | 171 |
| *GLI2* | AGCAGCAGCAACTGTCTGAGTGA<br>GACCTTGCTGCGCTTGTGAA | 60 | 105 |
| *GLI3* | TCCAACACAGAGGCCTATTCCAG<br>CTCTTGTTGTGCATCGGGTCA | 60 | 141 |
| *PTCH1b* | ACCAGAATGGGTCCACGACAA<br>AAAGTCTGAGGTGTCCCGCAA | 60 | 127 |
| *SMO* | GCCATGTTTGGAACTGGCATC<br>ATCCGCTTTGGCTCATCGTC | 58 | 119 |
| *GAPDH* | GAGTCAACGGATTTGGTCGT<br>GACAAGCTTCCCGTTCTCAG | 58 | 185 |

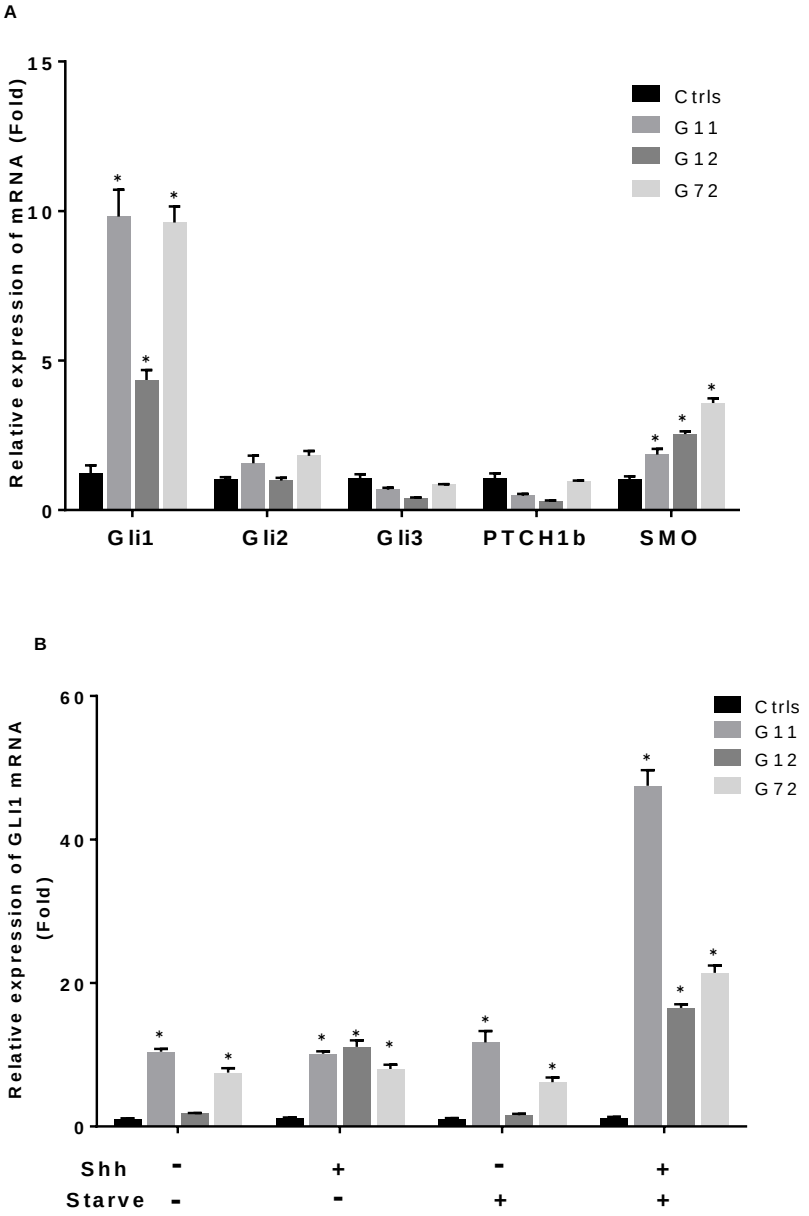



Fig. 1 Hedgehog signaling was activated in fibroblasts derived from patients with Gorlin syndrome. (A) The relative expression of the mRNA of hedgehog pathway-related proteins in control fibroblasts (Ctrls) and Gorlin syndrome fibroblasts (G11, G12, G72). (B) The relative expression of Gli1mRNA (mean ± SEM; **P*<0.05).

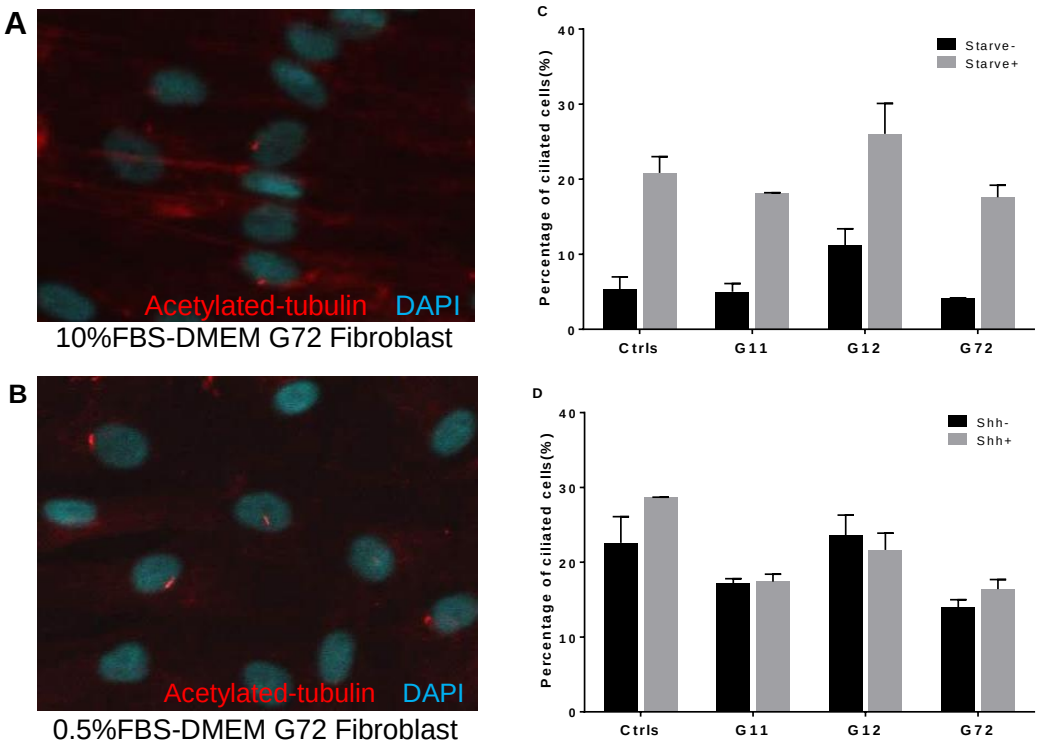



Fig. 2 The formation of primary cilia was increased after serum starvation in fibroblasts. (A) Immunostaining for primary cilia with acetylated tubulin (red) in GS fibroblasts without serum starvation and (B) 48hrs after serum removal. (C) Percentage of ciliated cells in control (Ctrl) and Gorlin syndrome (G-number) fibroblasts with (starvation-) or without serum (starvation+). (D) Percentage of ciliated cells with serum starvation and shh.

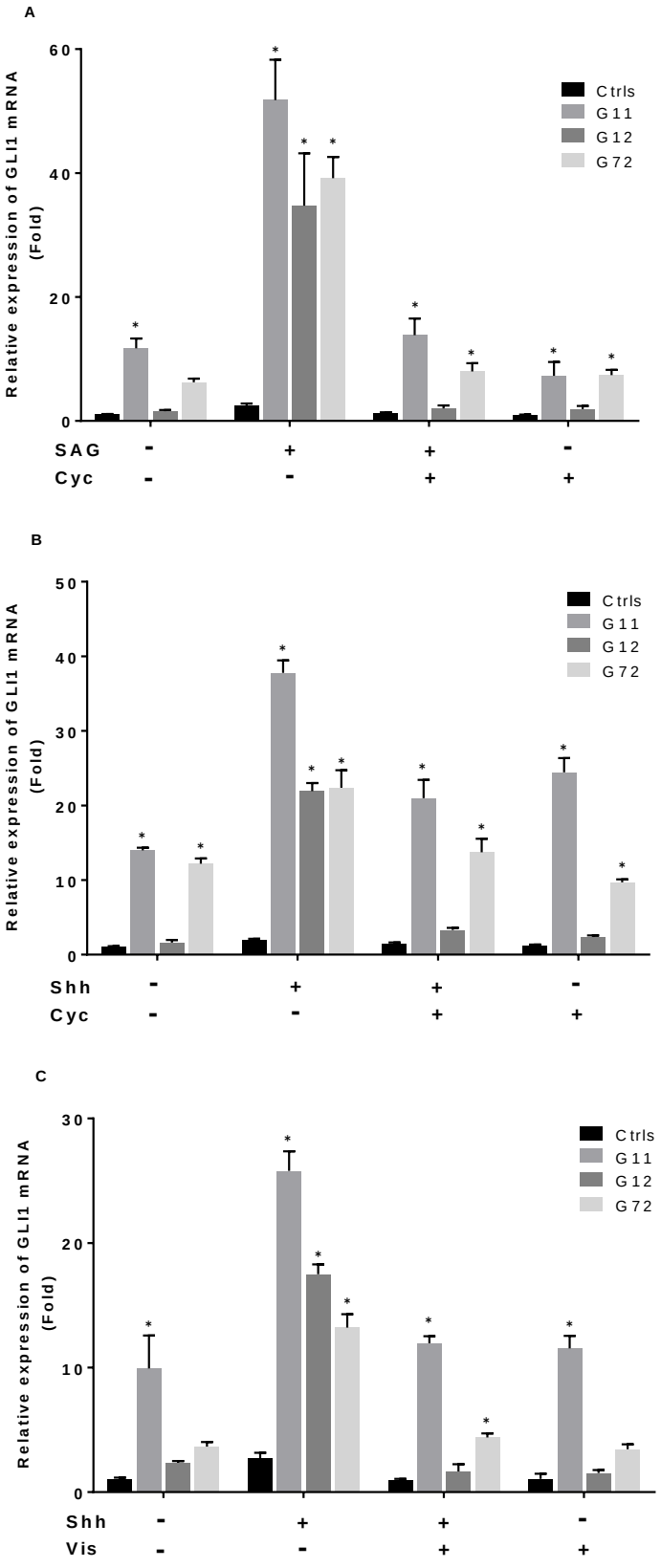
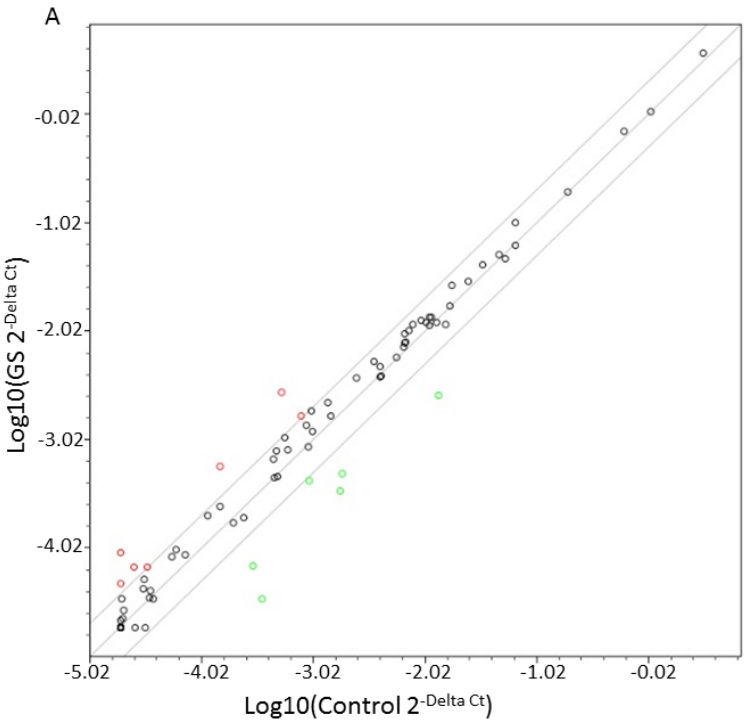



Fig. 3 Cyclopamine and vismodegib inhibited hedgehog signaling in GS fibroblasts. (A) The relative expression of GLI1 mRNA in control fibroblasts (Ctrls) and Gorlin syndrome fibroblasts (G-number) under the condition of serum starvation and treatment with shh and/or cyclopamine (Cyc). (B) The relative expression levels of GLI1 mRNA under the serum deprivation condition and treatment with the SMO agonist (SAG) and/or cyclopamine. (C) The relative expression levels of Gli1 mRNA under serum deprivation conditions and treatment with shh and/or vismodegib (mean ± SEM; **P*<0.05).




**B**

| Gene Symbol | Fold Regulation |
|---|---|
| GLI1 | 5.2091 |
| SMO | 2.1587 |
| WNT8B | 2.0719 |
| WNT9A | 2.6813 |
| ZIC1 | 3.8506 |
| ZIC2 | 2.5032 |

**C**

| Gene Symbol | Fold Regulation |
|---|---|
| BMP4 | -10.3108 |
| BMP8A | -4.2586 |
| FGF9 | -5.1773 |
| SFRP1 | -5.2025 |
| WNT16 | -3.6943 |
| WNT2B | -2.2354 |

Fig. 4. Analysis of the transcription of hedgehog pathway-related proteins.
(A) Scatterplots representing fold changes in mRNA levels in control fibroblasts and Gorlin syndrome fibroblasts under serum deprivation conditions with the shh treatment. Each circle shows a hedgehog-related gene. The central line represents no change; a circle above the central line shows a gene, the expression of which was increased: below shows a gene, the expression of which was decreased. Gray lines indicate 2-fold increases or decreases. (B) A table showing genes with expression levels that were increased by 2-fold and (C) decreased by 2-fold.

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