

RESEARCH ARTICLE

Counterregulatory roles of GLI2 and GLI3 in osteogenic differentiation via *Gli1* expressionYuto Takahashi^{1,§}, Yamato Ishida^{1,§}, Saishu Yoshida^{2,*}, Hye-Won Shin¹, Yohei Katoh^{1,‡} and Kazuhisa Nakayama^{1,¶}

ABSTRACT

The GLI1, GLI2 and GLI3 transcription factors mediate Hedgehog (Hh) signaling, which is crucial for bone development. During intramembranous ossification, mesenchymal stem cells (MSCs) are directly differentiated into osteoblasts. Under basal and Hh pathway-stimulated conditions, primary cilia play essential roles in proteolytic processing of GLI3 to its repressor form (GLI3R) and in activation of GLI2. Although previous studies in mice have suggested that *Gli1* expression depends on GLI2 and GLI3, coordinated roles of GLI1, GLI2 and GLI3 in osteogenic differentiation are not fully understood at the cellular level. From the MSC line C3H10T1/2, we established *Gli2*-knockout (KO) and *Gli3*-KO cells, as well as constitutively GLI3R-producing (cGLI3R) cells, and expressed GLI1, GLI2 and GLI3 constructs in these cell lines. The results demonstrate at the cellular level that GLI2 and GLI3R counterregulate osteogenic differentiation via activation and repression of *Gli1* expression, respectively; GLI3R, which results from GLI3 processing requiring protein kinase A-mediated phosphorylation, downregulates expression of *Gli2* as well as *Gli1*; and GLI1 upregulates expression of *Gli1* itself and *Gli2*, constituting a GLI1–GLI2 positive feedback loop.

KEY WORDS: Cilia, GLI1, GLI2, GLI3, Hedgehog signaling, Osteogenesis

INTRODUCTION

The Hedgehog (Hh) pathway is a signaling system that regulates multiple aspects of development, including cell proliferation, cell differentiation and tissue morphogenesis. Although this pathway is evolutionarily conserved in diverse organisms from *Drosophila* to humans, Hh signaling in vertebrates, but not in *Drosophila*, fully depends on primary cilia (Kopinke et al., 2021; Zhou and Jiang, 2022). Owing to the crucial roles of cilia in development and adult homeostasis, their dysfunction often causes congenital disorders with a broad spectrum of clinical features, which are collectively referred to as ciliopathies (Mill et al., 2023; Reiter and Leroux, 2017).

Three cilia-localized transmembrane proteins are crucial for Hh signaling; namely, the 12-pass transmembrane protein patched 1

(PTCH1), the class A G protein-coupled receptor (GPCR) GPR161 and the class F GPCR smoothened (SMO) (Anvarian et al., 2019; Kopinke et al., 2021). PTCH1 is a receptor for Hh ligands, and SMO and GPR161 regulate Hh signaling positively and negatively, respectively (Kopinke et al., 2021). On the other hand, the transcriptional output of Hh signaling is controlled by the glioma-associated oncogene (GLI) transcription factors (Niewiadomski et al., 2019). Although in *Drosophila* there is only one GLI protein, named Ci, and Hh signaling is independent of cilia, in vertebrates there are three GLI transcription factors (GLI1, GLI2 and GLI3) and Hh signaling is dependent on primary cilia (Hui and Angers, 2011). These GLI proteins have a zinc finger DNA-binding domain in the middle region and a C-terminal transactivation domain (Fig. 1A). In addition, GLI2 and GLI3 have an N-terminal repressor domain and a Suppressor of Fused (SUFU)-binding region within or near the repressor domain (Fig. 1A). As GLI1 lacks the repressor domain, it only acts as a transcriptional activator. On the other hand, GLI2 and GLI3 have been suggested to have both activator and repressor functions (Hui and Angers, 2011; Niewiadomski et al., 2019). In the off state of the Hh pathway, PTCH1 suppresses SMO by inhibiting its entry into cilia, and GPR161 on the ciliary membrane activates protein kinase A (PKA) to induce the phosphorylation of GLI2 and GLI3 (Fig. 1A). This results in the partial proteolytic processing of GLI3 to yield its repressor form, GLI3R (Niewiadomski et al., 2014; Wang et al., 2000, 2007), whereas the processing of GLI2 to GLI2R is thought to be inefficient (Pan and Wang, 2007). In the on state, binding of Hh ligands to PTCH1 triggers its removal from cilia, relieves SMO inhibition to enable its entry into cilia and leads to the concomitant removal of GPR161 from cilia, resulting in cancellation of the PKA-mediated negative regulation of the signaling (Mukhopadhyay et al., 2013). Studies using knockout (KO) and knock-in mice have therefore suggested that the expression of Hh target genes is likely to be determined by the balance between GLI3R and the activator form of GLI2 (Kopinke et al., 2021).

Ciliopathies often accompany skeletal and craniofacial dysplasia, indicating the importance of primary cilia in skeletal development (Chinipardaz et al., 2022; Mill et al., 2023; Quadri and Upadhyai, 2023). During skeletal development, bone formation takes place in two distinct processes: intramembranous ossification and endochondral ossification (Salhotra et al., 2020). New bones that are added to the outer surface of long bones and flat bones of the skull are generated by intramembranous ossification, in which mesenchymal stem cells (MSCs) are directly differentiated into osteoblasts. During endochondral ossification, MSCs develop into chondrocytes, around which the perichondrium is formed. Osteoblasts are then generated from the perichondrium under the influence of chondrocytes. The Hh signaling pathway plays a vital role in the process of bone formation, such as in differentiation of the osteoblast lineage (Salhotra et al., 2020; Zhou et al., 2022a). GLI2 and GLI3 are essential for normal skeletal development (Mo et al., 1997; Rice et al., 2010). Dominant

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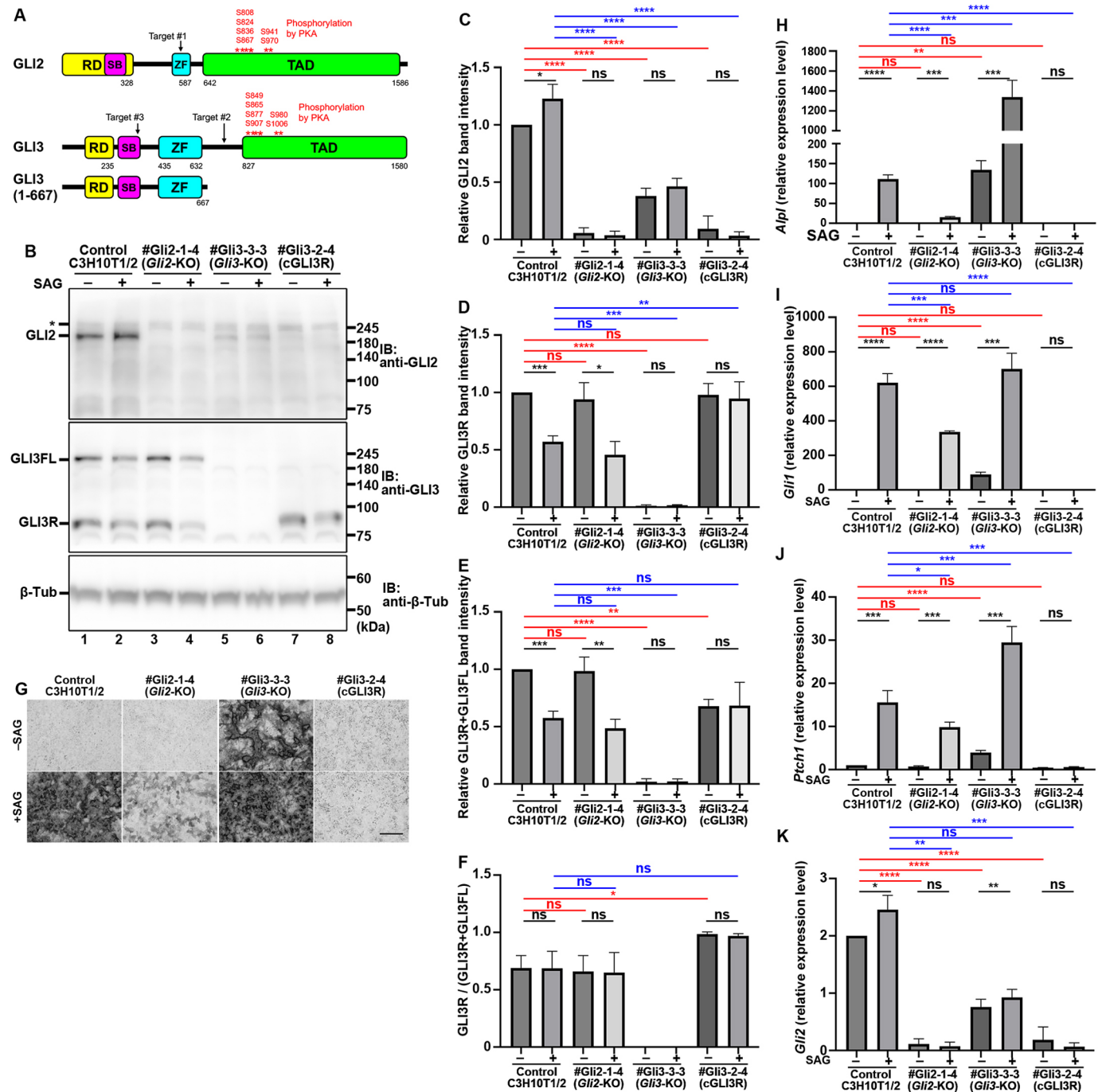


Fig. 1. Defects of osteogenic differentiation and Hh signaling in *Gli2*-KO, *Gli3*-KO and cGLI3R C3H10T1/2 cells. (A) Schematic representation of the domain organization of GLI2 and GLI3, and that of the GLI3(1–667) construct. PKA phosphorylation sites are indicated by asterisks. Positions of target sequences for the disruption of *Gli2* and *Gli3* genes are indicated by arrows. RD, repressor domain; SB, SUFU-binding region; TAD, transactivation domain; ZF, zinc finger domain. Numbers indicate amino acid positions. (B–K) Control C3H10T1/2 cells or #Gli2-1-4, #Gli3-3-3 or #Gli3-2-4 cells were incubated in the absence (–SAG) or presence (+SAG) of 10 nM SAG for 7 days. (B) Lysates prepared from the cells were subjected to SDS-PAGE and immunoblotting (IB) analysis using an anti-GLI2, anti-GLI3 or anti-β-tubulin (β-Tub) antibody. The asterisk indicates the position of nonspecific bands. (C–F) Band intensities of GLI2 (C), GLI3R (D), total GLI3 (GLI3FL+GLI3R) (E) and the ratio of GLI3R to total GLI3 (F) were compared. (G) The cells were stained for ALP activity. Scale bar: 250 μm. Images are representative of three experiments. (H–K) RNA extracted from the cells was subjected to RT-qPCR for *Alpl* (H), *Gli1* (I), *Ptc1* (J) or *Gli2* (K) transcripts. In C–F and H–K, data are shown as the mean±s.d. of triplicates. Statistical significances among multiple groups and those between –SAG and +SAG were calculated using one-way ANOVA followed by Dunnett's multiple comparison test and two-tailed unpaired Student's *t*-test, respectively. *****P*<0.0001; ****P*<0.001; ***P*<0.01; **P*<0.05; ns, not significant.

variants of *GLI3* are known to cause ciliopathies, namely Greig cephalopolysyndactyly syndrome (GCPS) and Pallister–Hall syndrome (PHS), which demonstrate canonical features of preaxial

polydactyly, and mesoaxial polydactyly and hypothalamic hamartoma, respectively (Johnston et al., 2005, 2010). In particular, PHS-type variants are expected to produce GLI3R-associated proteins,

whereas GCPS is probably caused by functional haploinsufficiency of *GLI3* (Johnston et al., 2005).

The mouse MSC line C3H10T1/2 has been used as a model for Hh-mediated osteogenic differentiation, as the stimulation of C3H10T1/2 cells with Hh ligands or SMO agonist (SAG) induces their direct differentiation into alkaline phosphatase (ALP)-positive osteoblasts (Nakamura et al., 1997; Ruiz i Altaba, 1999; Shimoyama et al., 2007; Yang and Wang, 2012). However, how *GLI1*, *GLI2* and *GLI3* are involved in the osteogenic differentiation of MSCs has remained unclear. From C3H10T1/2 cells, we here established not only *Gli2*-KO and *Gli3*-KO cell lines, but also a cell line that constitutively produces a *GLI3R*-associated protein. Analysis using these cell lines unequivocally demonstrated that *GLI2* and *GLI3* have counterregulatory roles in osteogenic differentiation via the expression of *Gli1*.

RESULTS

Constitutive *GLI3R* production suppresses *GLI1* and *GLI2* expression and inhibits osteogenic differentiation

To investigate the roles of *GLI2* and *GLI3* in the differentiation of MSCs into osteoblasts, we disrupted the *Gli2* and *Gli3* genes in C3H10T1/2 cells using the CRISPR/Cas9 system by homology-independent DNA repair (Katoh et al., 2017). Target sequence #1, which was designed to disrupt the *Gli2* gene, is located within the zinc finger domain (Fig. 1A). For disruption of the *Gli3* gene, two target sequences were selected; target sequence #2 is located between the zinc finger domain and the transactivation domain, and target sequence #3 is located within the SUFU-binding region (Fig. 1A). Immunoblotting analysis of cell lysates demonstrated that the treatment of control C3H10T1/2 cells with SAG slightly but significantly increased the *GLI2* protein level (Fig. 1B, top panel, lanes 1 and 2; also see Fig. 1C); it is also of note that a protein band corresponding to *GLI2R* was not detectable (Fig. 1B, top panel, lanes 1 and 2). On the other hand, protein bands corresponding to full-length *GLI3* (*GLI3FL*) and *GLI3R* were detected by immunoblotting analysis of C3H10T1/2 cell lysates (Fig. 1B, middle panel, lanes 1 and 2). Upon SAG treatment, the levels of *GLI3R* protein and total *GLI3* proteins (*GLI3R*+*GLI3FL*) were decreased (Fig. 1D,E) although the ratio *GLI3R*/(*GLI3R*+*GLI3FL*) was not significantly changed (Fig. 1F).

For the established #*Gli2*-1-4 cell line (Fig. S1A,B), we could not detect the *GLI2* protein by immunoblotting analysis (Fig. 1B, top panel, lanes 3 and 4; also see Fig. 1C). Although we could detect one disrupted *Gli2* sequence (Fig. S1A,B), our data indicate that the *Gli2* alleles in the #*Gli2*-1-4 cell line are likely disrupted, so that they do not produce the *GLI2* protein. Results of the analysis of cell differentiation also supported that the *Gli2* alleles are disrupted in the #*Gli2*-1-4 cell line (see below; Fig. 1G). Although we do not know the exact reason why only one disrupted allele was detected in the #*Gli2*-1-4 cell line, it is possible that a large deletion occurred in the other *Gli2* alleles such that a set of primers to detect disruption of the *Gli2* gene could not bind, and/or that the same 1 bp insertion occurred in multiple *Gli2* alleles.

It is also of note that in the #*Gli2*-1-4 cell line, neither the levels of the *GLI3R* protein and total *GLI3* proteins (*GLI3R*+*GLI3FL*) nor the ratio *GLI3R*/(*GLI3R*+*GLI3FL*) were significantly changed, compared with control C3H10T1/2 cells (Fig. 1D–F). These results indicate that *GLI2* does not affect the expression level of *GLI3* or proteolytic processing of *GLI3FL* to *GLI3R*.

The *Gli3* locus on chromosome 13 in C3H10T1/2 cells is likely to be at least triploid, as three disrupted alleles were detected for both the target #2 and target #3 cell lines (Fig. S1C–F). For the #*Gli3*-3-3

cell line, no *GLI3* protein was detected (Fig. 1B, middle panel, lanes 5 and 6; also see Fig. 1E). By contrast, for the #*Gli3*-2-4 cell line, an intense band in the position corresponding to *GLI3R* was detected, whereas a band for *GLI3FL* was not detected (Fig. 1B, middle panel, lanes 7 and 8). Thus, a *GLI3R*-associated protein is constitutively produced in the #*Gli3*-2-4 cell line. In this context, it is interesting to note that the *GLI2* protein was not detected in the #*Gli3*-2-4 cell line by immunoblotting analysis (Fig. 1B, top panel, lanes 7 and 8). The reduction in *GLI2* protein level in #*Gli3*-2-4 cells was owing to a reduction in the level of the *Gli2* transcript (see Fig. 1K).

GLI2 and *GLI3* are known to become enriched at the ciliary tip when the Hh pathway is stimulated (for example, see Fig. S3A,E and Fig. S3I,M; also see Fig. S3Q,R) (Endoh-Yamagami et al., 2009; Kim et al., 2009; Tukachinsky et al., 2010; Wen et al., 2010). Consistent with the immunoblotting data, *GLI2* was not detectable at the ciliary tip even upon SAG treatment in #*Gli3*-2-4 cells as well as in #*Gli2*-1-4 cells (Fig. S3B,D,F,H; also see Fig. S3Q) owing to lack of the *GLI2* protein expression in these cells (Fig. 1B, top panel, lanes 3, 4, 7, and 8), whereas the level of *GLI2* in the ciliary tip of #*Gli3*-3-3 cells was comparable to that of control C3H10T1/2 cells (Fig. S3C,G; also see Fig. S3Q). Taken together with the immunoblotting data (Fig. 1B), the constitutively produced *GLI3R*-associated protein in #*Gli3*-2-4 cells is likely to suppress the expression of *Gli2* (also see Fig. 1G). On the other hand, *GLI3* level at the ciliary tip upon SAG stimulation was not substantially affected in #*Gli2*-1-4 cells (Fig. S3J,N; also see Fig. S3R). In addition, *GLI3* was not detectable at the tip in #*Gli3*-3-3 or #*Gli3*-2-4 cells under basal or SAG-stimulated conditions (Fig. S3K,L,O,P; also see Fig. S3R). We hereafter refer to #*Gli2*-1-4, #*Gli3*-3-3 and #*Gli3*-2-4 cells as *Gli2*-KO, *Gli3*-KO, and c*GLI3R* (for constitutively *GLI3R*-producing) cells, respectively.

We then investigated the ability of these established cell lines to differentiate into ALP-positive osteoblasts. When treated with SAG, control C3H10T1/2 cells differentiated into cells that were positively stained for ALP activity (Fig. 1G; left column). In the case of *Gli2*-KO cells, the ALP signal was only slightly increased by SAG treatment (Fig. 1G, second column). In clear contrast, virtually no differentiation into ALP-positive cells was observed upon SAG treatment of c*GLI3R* cells (Fig. 1G, fourth column). On the other hand, the differentiation of *Gli3*-KO cells into ALP-positive cells was observed even in the absence of SAG treatment (Fig. 1G, third column). The cytochemical ALP staining data were confirmed by reverse transcription-quantitative PCR (RT-qPCR) analysis of the transcript of *Alpl* (biomineralization-associated ALP). As shown in Fig. 1H, SAG treatment increased the *Alpl* transcript level by ~100-fold in control C3H10T1/2 cells. In striking contrast, virtually no *Alpl* transcript was detected in c*GLI3R* cells, whereas in *Gli3*-KO cells, the basal *Alpl* transcript level was more than 100-fold higher than that in control cells, and the SAG-stimulated *Alpl* level was more than 1000-fold higher. On the other hand, in *Gli2*-KO cells, although the basal *Alpl* level was comparable to that in control cells, the *Alpl* level upon SAG stimulation was approximately a tenth of that in control cells. Therefore, the absence of *GLI3* and/or *GLI3R* promotes the differentiation of C3H10T1/2 cells into ALP-positive cells, whereas the absence of *GLI2* suppresses the differentiation.

Similar results were obtained when these cells were treated with sonic hedgehog (Shh), instead of SAG. Treatment of control C3H10T1/2 cells with Shh led to their differentiation into ALP-positive cells (Fig. S2A, left column), concomitant with a more than 1000-fold increase in the *Alpl* transcript level (Fig. S2B). By contrast, the Shh-induced differentiation of *Gli2*-KO and c*GLI3R* cells into ALP-positive cells (Fig. S2A, second and fourth columns, respectively) and increase in the *Alpl* transcript level in these

cells (Fig. S2B) were compromised. In addition, differentiation of *Gli3*-KO cells was high under basal conditions and further increased by the Shh treatment (Fig. S2A, third column, and Fig. S2B).

It is also known that MSCs can be differentiated into osteoblasts by culturing them in a medium containing ascorbic acid and β -glycerophosphate (Joung et al., 2012; Shi et al., 2015). When control C3H10T1/2 cells were cultured in the presence of ascorbic acid and β -glycerophosphate, the *Alpl* transcript level increased more than 30-fold (Fig. S2E), although ALP staining intensity was only slightly increased (Fig. S2D, left column). Thus, C3H10T1/2 cells treated with ascorbic acid and β -glycerophosphate were confirmed to undergo osteogenic differentiation, although not to the same extent as those treated with SAG or Shh. By contrast, the increase in the *Alpl* transcript level was significantly suppressed in *Gli2*-KO and cGLI3R cells compared with that in control cells (Fig. S2E). Differentiation of *Gli3*-KO cells was high in the basal state and was further increased by the treatment with ascorbic acid and β -glycerophosphate (Fig. S2D, third column, and Fig. S2E).

We also analyzed the levels of *Gli1* and *Ptch1* transcripts. In cGLI3R cells, virtually no increase in *Gli1* or *Ptch1* level was observed upon SAG treatment (Fig. 1I,J). In *Gli3*-KO cells, basal *Gli1* and *Ptch1* levels were higher than those in control C3H10T1/2 cells, and SAG treatment further increased *Gli1* and *Ptch1* transcript levels (Fig. 1I,J). In *Gli2*-KO cells, SAG treatment increased the levels of the *Gli1* and *Ptch1* transcripts to some extent, albeit not as much as in control C3H10T1/2 cells (Fig. 1I,J). Similar results were obtained for the *Gli1* transcript level when these cells were treated with Shh instead of SAG (compare Fig. S2C with Fig. 1I). Thus, the data shown in Fig. 1H–J and Fig. S2A–C indicate that GLI3 functions primarily as a transcriptional repressor in the basal state and GLI2 functions as a transcriptional activator upon Hh pathway stimulation. However, as proposed in previous studies using mice with *Gli2*^{−/−}, *Gli3*^{−/−} and *Gli2*^{−/−} *Gli3*^{−/−} alleles (Buttitta et al., 2003; Kesper et al., 2010; Mo et al., 1997; Motoyama et al., 2003), GLI3 might be able to act as a transcriptional activator to induce *Gli1* expression in the absence of GLI2 (see below).

On the other hand, treatment of cells with ascorbic acid and β -glycerophosphate produced clearly different results from those of SAG and Shh with respect to the *Gli1* level. Namely, in all cells examined (control C3H10T1/2, *Gli2*-KO, *Gli3*-KO and cGLI3R cells), the treatment with ascorbic acid and β -glycerophosphate significantly reduced the *Gli1* transcript level from the basal level (Fig. S2F). Thus, the fact that, despite the decreased *Gli1* level, osteogenic differentiation as indicated by the increased ALP staining and *Alpl* level is promoted indicates that the treatment of C3H10T1/2 cells with ascorbic acid and β -glycerophosphate promotes osteogenic differentiation by a mechanism different from that of SAG and Shh (see Discussion).

We further analyzed *Gli2* transcript levels, as the GLI2 protein was not detectable in cGLI3R cells (Fig. 1B, top panel, lanes 7 and 8). As shown in Fig. 1K, the *Gli2* transcript could not be detected in cGLI3R cells, whereas the *Gli2* transcript level in *Gli3*-KO cells was not substantially different from that in control C3H10T1/2 cells; note that the *Gli2* transcript was detected even in the case of *Gli2*-KO cells, as the PCR primer sequences used were located to the 5' side of the target sequence used to disrupt the *Gli2* gene. To the best of our knowledge, this is the first line of evidence directly showing that GLI3R suppresses the expression of *Gli2* as well as *Gli1* and *Ptch1*, although a previous study using chromatin immunoprecipitation has indicated the direct binding of GLI3 to target sequences in the 5' flanking region of not only *Gli1* but also *Gli2* in mouse kidney tissue cultured in the presence of cyclopamine (Hu et al., 2006). The

data on *Gli2*-KO, *Gli3*-KO, and cGLI3R cells are compatible with the proposed model in which GLI3 functions primarily as a transcriptional repressor and its deficiency results in gain-of-function phenotypes, whereas GLI2 in practice functions as a transcription activator (Niewiadomski et al., 2019).

GLI2 promotes the osteogenic differentiation of C3H10T1/2 cells upon Hh pathway stimulation

We then investigated whether the defect in the differentiation of *Gli2*-KO cells into ALP-positive cells could be rescued by the exogenous expression of GLI2. In addition to human wild-type GLI2 [GLI2(WT)], we also used 6SA and 6SD GLI2 mutants, in which six Ser residues that undergo PKA-mediated phosphorylation (see Fig. 1A) (Niewiadomski et al., 2014; Wang et al., 2000) are replaced with either Ala to block phosphorylation or by Asp to mimic the phosphorylated state, respectively. The stable expression of EGFP-fused GLI2(WT), GLI2(6SA) and GLI2(6SD) in *Gli2*-KO cells was confirmed by immunoblotting analysis of the cell lysates (Fig. 2A, top panel, lanes 5–10).

The cells were stained for ALP activity. ALP staining under SAG-stimulated conditions was increased by the expression of any of the GLI2 constructs, compared with that of cells expressing EGFP alone (Fig. 2B, lower panels; compare columns 3–5 with column 2). In the case of *Gli2*-KO cells expressing EGFP-fused GLI2(6SA) or GLI2(6SD), substantial ALP staining under basal conditions was observed, compared with that of *Gli2*-KO cells expressing EGFP–GLI2(WT) (Fig. 2B, upper panels; compare columns 4 and 5 with column 3).

ALP staining data were confirmed by RT-qPCR of the *Alpl* transcript. As shown in Fig. 2C, *Alpl* transcript levels in *Gli2*-KO cells expressing EGFP alone were substantially lower under both basal and SAG-stimulated conditions than in control C3H10T1/2 cells. In good agreement with the ALP staining data, *Alpl* transcript levels were restored by the expression of EGFP–GLI2(WT), EGFP–GLI2(6SA) or EGFP–GLI2(6SD); in particular, the *Alpl* transcript level in the basal state was significantly higher in *Gli2*-KO cells expressing GLI2(6SA) or GLI2(6SD) than in those expressing GLI2(WT). These results thus indicate that GLI2 acts as an activator of *Alpl* expression upon Hh pathway stimulation. These results also suggest that PKA-mediated phosphorylation of GLI2 in the basal state suppresses its function in some way, and thereby the substitution of Ser residues that are able to be phosphorylated can convert the GLI2 protein into an activated form. In addition, it is of note that although we used GLI2(6SD) to mimic the phosphorylated state, this GLI2 mutant might have similar properties to GLI2(6SA), which cannot be phosphorylated.

We also investigated the levels of *Gli1* and *Ptch1* transcripts in *Gli2*-KO cells expressing the GLI2 constructs. *Gli1* and *Ptch1* levels were increased in cells expressing GLI2(6SA) or GLI2(6SD) compared with those in GLI2(WT)-expressing cells, but the changes were small compared with the changes in *Alpl* level under either basal or SAG-stimulated conditions (Fig. 2D,E). These results are thus consistent with the previous view that in the absence of endogenous GLI2, GLI3 may act as a transcriptional activator for the expression of *Gli1* (also see Fig. 5D,E) (Buttitta et al., 2003; Mo et al., 1997; Motoyama et al., 2003), and that the effects of additional GLI2 expression are minimal.

We then investigated the localization of the EGFP–GLI2 constructs. EGFP–GLI2(WT) signals were detected at low levels at the ciliary tip in the absence of SAG, and the level was increased by the treatment of cells with SAG (Fig. S4B,F,I; Fig. S4A,E, negative controls), which is consistent with previous studies showing that GLI2 is recruited to the ciliary tip upon Hh pathway

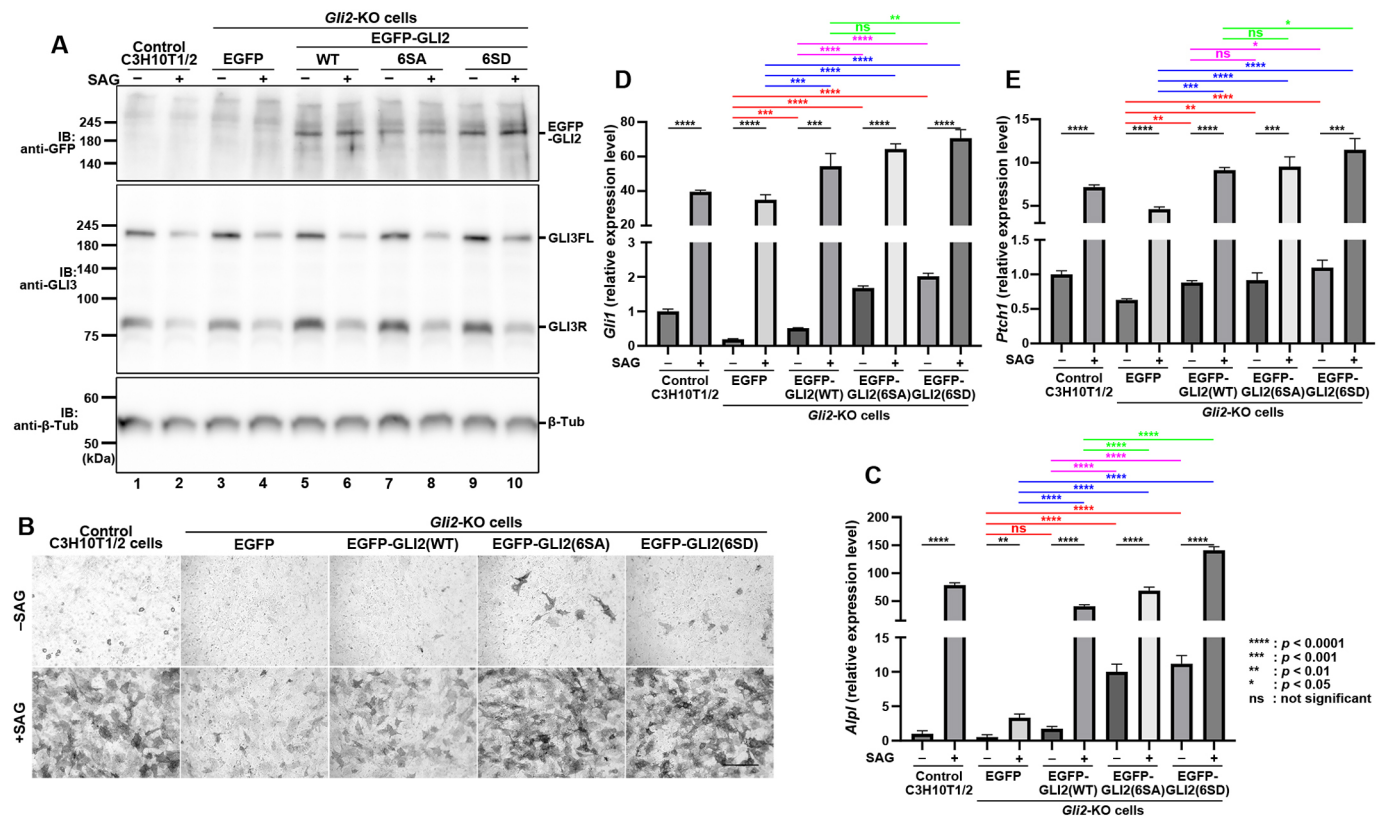


Fig. 2. Phosphorylation site mutations of GLI2 mildly affect osteogenic differentiation and *Gli1* expression under basal conditions. Control C3H10T1/2 cells or *Gli2*-KO cells (#Gli2-1-4) stably expressing EGFP or EGFP-fused GLI2(WT), GLI2(6SA) or GLI2(6SD) were incubated in the absence (–SAG) or presence (+SAG) of 10 nM SAG for 7 days. (A) Lysates prepared from the cells were subjected to SDS-PAGE and immunoblotting (IB) analysis using an anti-GLI2, anti-GLI3 or anti-β-tubulin (β-Tub) antibody. Blots shown are representative of three experiments. (B) The cells were stained for ALP activity. Scale bar: 250 μm. Images shown are representative of two experiments. (C–E) RNA extracted from the cells was subjected to RT-qPCR for *Alpl* (C), *Gli1* (D) or *Ptc1* (E) transcripts. Data are shown as the mean±s.d. of triplicates. Statistical significances among multiple groups and those between –SAG and +SAG were calculated using one-way ANOVA followed by Dunnett's multiple comparison test and two-tailed unpaired Student's *t*-test, respectively.

stimulation (Kim et al., 2009; Tukachinsky et al., 2010). The basal level of EGFP–GLI2(6SA) at the tip of cilia was substantially higher than that of EGFP–GLI2(WT), but the level was not changed by SAG treatment (Fig. S4C,G,I). The basal level of EGFP–GLI2(6SD) at the tip of cilia also had a tendency to be higher than that of EGFP–GLI2(WT), although the difference was not significant (Fig. S4D,H,I). Thus, as discussed above, phosphorylation of GLI2 by PKA might suppress not only its function but also its translocation to the ciliary tip under basal conditions, although the underlying mechanism remains unknown.

GLI3 inhibits osteogenic differentiation under basal conditions by suppressing *Gli1* and *Gli2* expression

We then investigated whether the constitutive differentiation of *Gli3*-KO cells into ALP-positive cells can be changed by the exogenous expression of GLI3 constructs. For these experiments, we used human wild-type GLI3 [GLI3(WT)], GLI3(6SA), GLI3(6SD) and GLI3(1–667), the last of which mimics the GLI3R form (see Fig. 1A). The stable expression of EGFP-fused GLI3(WT), GLI3(6SA), GLI3(6SD) and GLI3(1–667) was confirmed by immunoblotting analysis of the cell lysates, although the expression levels of EGFP–GLI3(6SA) and EGFP–GLI3(6SD) appeared to be lower than the expression level of EGFP–GLI3(WT) (Fig. 3A, top panel, lanes 5–12). It is interesting to note that the band corresponding to EGFP–GLI3R was detected in EGFP–GLI3(WT)-expressing cells but not in EGFP–GLI3(6SA)-expressing or EGFP–GLI3(6SD)-

expressing cells, indicating that the substitution of Ser residues that are able to be phosphorylated by PKA can block the processing of GLI3FL into GLI3R. Again, GLI3(6SD) probably acted as a nonphosphorylatable mutant rather than a phosphorylation-mimicking one. It is also noteworthy that the expression of EGFP–GLI3(WT) as well as EGFP–GLI3(1–667) reduced the GLI2 protein level (Fig. 3A, middle panel, compare lanes 5, 6, 11 and 12 with lanes 3 and 4; also see Fig. 3F).

The constitutive differentiation of *Gli3*-KO cells into ALP-positive cells was substantially suppressed by the stable expression of not only EGFP–GLI3(1–667) but also EGFP–GLI3(WT) under both basal and SAG-stimulated conditions (Fig. 3B, compare columns 3 and 6 with column 2; also see Fig. 3C). On the other hand, when EGFP-fused GLI3(6SA) or GLI3(6SD) was expressed in *Gli3*-KO cells, the cells remained differentiated (Fig. 3B, columns 4 and 5). The ALP staining data were then confirmed by RT-qPCR of the *Alpl* transcript. As shown in Fig. 3C, the increase in *Alpl* transcript levels under both basal and SAG-stimulated conditions in *Gli3*-KO cells (expressing EGFP alone) were significantly suppressed by the expression of EGFP–GLI3(WT) as well as the expression of EGFP–GLI3(1–667). By contrast, the expression of EGFP–GLI3(6SA) further increased the basal and SAG-stimulated levels of the *Alpl* transcript, compared with *Gli3*-KO cells expressing EGFP alone, which was compatible with the results of a previous study using knock-in mice (Wang et al., 2007). The expression of EGFP–GLI3(6SD) also had a tendency to increase *Alpl* transcript levels, although the changes were not statistically

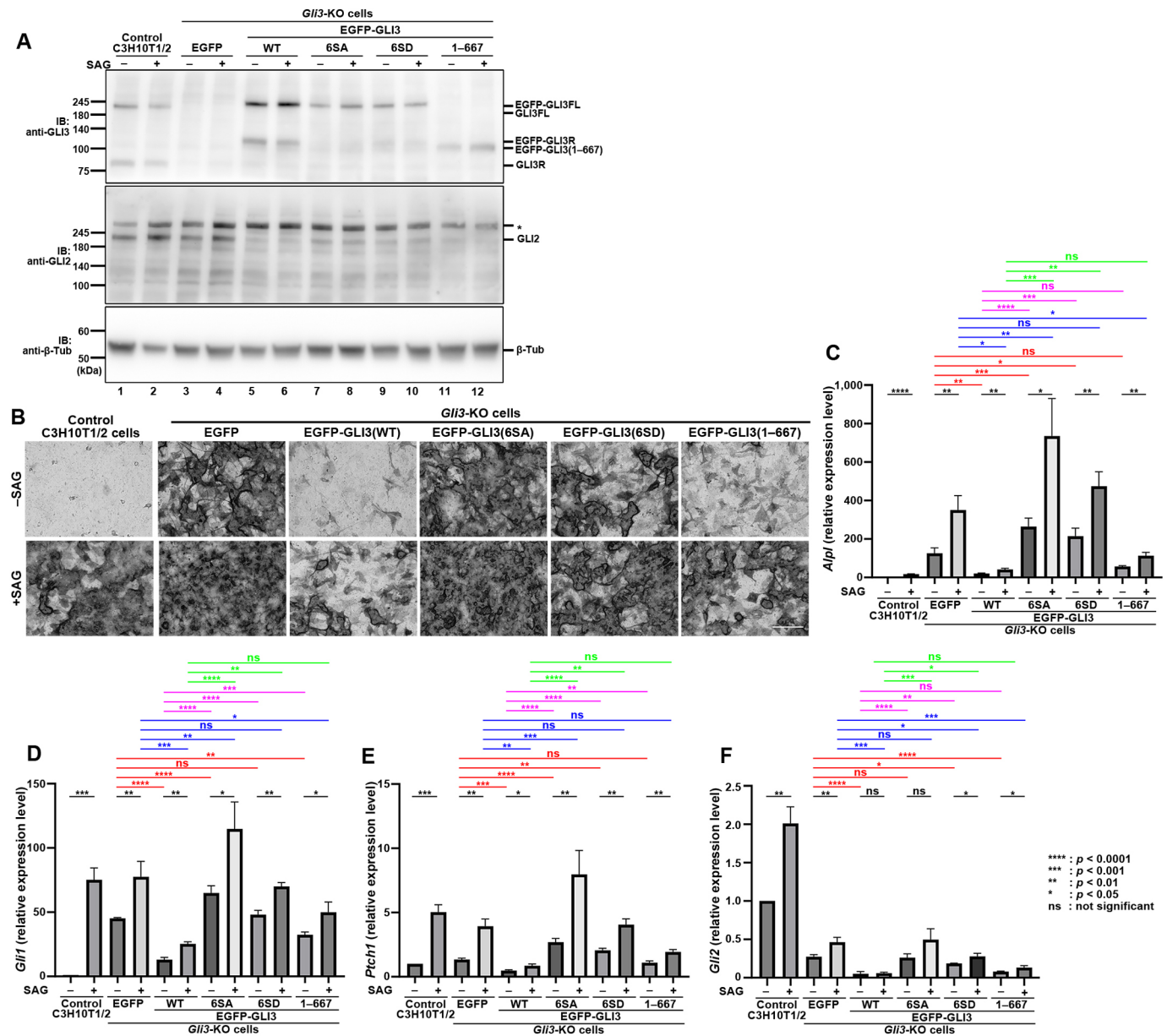


Fig. 3. Phosphorylation site mutations of GLI3 impair its proteolytic processing and cause constitutive osteogenic differentiation and Hh signaling activation. Control C3H10T1/2 cells or *Gli3*-KO cells (#Gli3-3-3) stably expressing EGFP or EGFP-fused GLI3(WT), GLI3(6SA), GLI3(6SD) or GLI3(1–667) were incubated in the absence (–SAG) or presence (+SAG) of 10 nM SAG for 7 days. (A) Lysates prepared from the cells were subjected to SDS-PAGE and immunoblotting (IB) analysis using an anti-GLI2, anti-GLI3 or anti-β-tubulin (β-Tub) antibody. The asterisk indicates the position of nonspecific bands. Blots shown are representative of three experiments. (B) The cells were stained for ALP activity. Scale bar: 250 μm. Images shown are representative of three experiments. (C–F) RNA extracted from the cells was subjected to RT-qPCR for the *Alpl* (C), *Gli1* (D), *Ptch1* (E) or *Gli2* (F) transcripts. Data are shown as the mean±s.d. of triplicates. Statistical significances among multiple groups and those between –SAG and +SAG were calculated using one-way ANOVA followed by Dunnett's multiple comparison test and two-tailed unpaired Student's *t*-test, respectively.

significant (Fig. 3C). These results indicate that GLI3(WT) acts predominantly as a repressor of differentiation into ALP-positive cells via processing into the GLI3R form. By contrast, taken together with the data in Fig. 3A (lanes 7–10), GLI3(6SA) and GLI3(6SD) are likely to function only as activators, as they lack processing to GLI3R.

The trend in the expression levels of *Gli1* and *Ptch1* transcripts in *Gli3*-KO cells expressing various GLI3 constructs (Fig. 3D,E) was similar to that of the expression level of *Alpl* (Fig. 3C), although the inhibition of *Ptch1* expression by GLI3(1–667) was not statistically significant. We also analyzed the *Gli2* transcript level, as the GLI2 protein level was substantially decreased in *Gli3*-KO cells expressing EGFP-fused GLI3(WT) or GLI3(1–667) compared

with that in control C3H10T1/2 cells and *Gli3*-KO cells expressing EGFP alone (Fig. 3A, middle panel). Consistent with the data of Fig. 3A (middle panel), the *Gli2* transcript level was significantly decreased by the exogenous expression of EGFP-fused GLI3(WT) or GLI3(1–667) (Fig. 3F).

Taken together with the phenotypic differences between *Gli3*-KO and cGLI3R cells (Fig. 1), these results indicate that GLI3 functions as a repressor via its processing into the GLI3R form, and that GLI3R suppresses the expression of not only *Gli1* but also *Gli2*.

We then investigated the localization of the EGFP–GLI3 constructs. A low level of EGFP–GLI3(WT) was found at the ciliary tip in the

basal state, which tended to increase under SAG-stimulated conditions (Fig. S5B,G,K; Fig. S5A,E, negative controls). The basal level of EGFP–GLI3(6SA) at the ciliary tip was higher than that of EGFP–GLI3(WT), and the level was further increased by SAG treatment (Fig. S5C,H,K). In the case of EGFP–GLI3(6SD), the basal and SAG-stimulated levels at the ciliary tip were not significantly different from those of EGFP–GLI3(WT) (Fig. S5D,I,K). In clear contrast, EGFP–GLI3(1–667) was not detected at the ciliary tip under both the basal and SAG-stimulated conditions (Fig. S5E,J,K), in agreement with previous observations that a full-length GLI3 construct is found at the ciliary tip, whereas a GLI3R construct is not detected at the ciliary tip but in the nucleus (Haycraft et al., 2005).

GLI3R overrides GLI3FL to suppress Hh signaling and osteogenic differentiation

The above data suggest the possibility that GLI3 predominantly acts as a repressor, even when a substantial amount of GLI3FL is present. The situation is reminiscent of individuals with PHS, who have one normal *GLI3* allele and one GLI3R-associated allele (Johnston et al., 2005, 2010). To support this possibility, we expressed GLI3(1–667) in control C3H10T1/2 cells, and GLI3(WT) in cGLI3R cells. Immunoblotting analysis of the cell lysates confirmed the expression of EGFP–GLI3(1–667) in control C3H10T1/2 cells (Fig. 4A, top panel, lanes 3 and 4) and that of EGFP–GLI3(WT) in cGLI3R cells (lanes 7 and 8); again, a substantial fraction of exogenously expressed

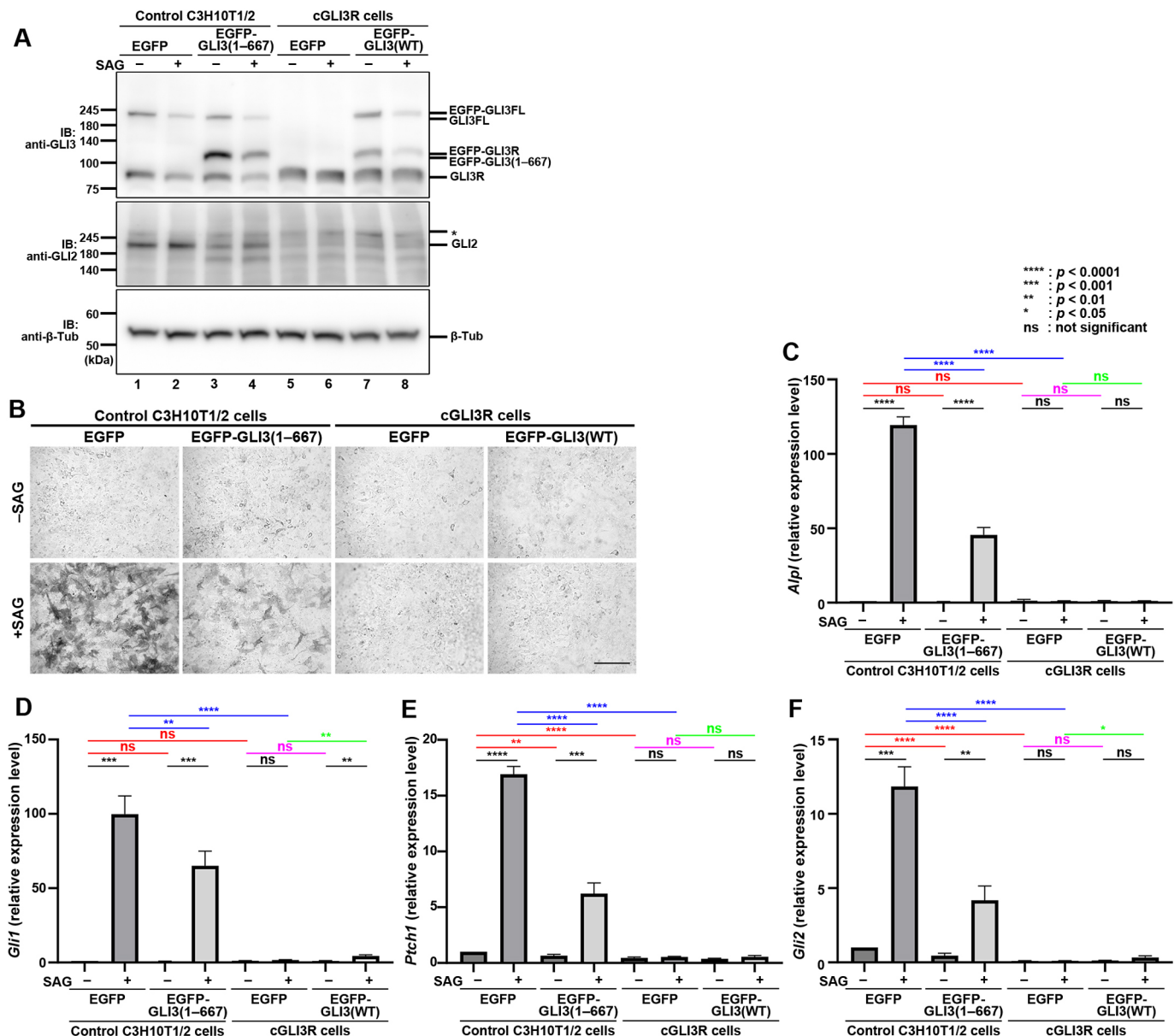


Fig. 4. Dominant effect of GLI3R on osteogenic differentiation and Hh signaling. Control C3H10T1/2 cells stably expressing EGFP or EGFP–GLI3(1–667), or cGLI3R cells (#Gli3-2–4) stably expressing EGFP or EGFP–GLI3(WT) were incubated in the absence (–SAG) or presence (+SAG) of 10 nM SAG for 7 days. (A) Lysates prepared from the cells were subjected to SDS-PAGE and immunoblotting (IB) analysis using an anti-GLI2, anti-GLI3, or anti-β-tubulin (β-Tub) antibody. The asterisk indicates the position of nonspecific bands. Blots shown are representative of two experiments. (B) The cells were stained for ALP activity. Scale bar: 250 μm. Images shown are representative of three experiments. (C–F) RNA extracted from the cells was subjected to RT-qPCR for the *Alpl* (C), *Gli1* (D), *Ptch1* (E) or *Gli2* (F) transcripts. Data are shown as the mean±s.d. of triplicates. Statistical significances among multiple groups and those between –SAG and +SAG were calculated using one-way ANOVA followed by Dunnett's multiple comparison test and two-tailed unpaired Student's *t*-test, respectively.

EGFP–GLI3(WT) was processed to EGFP–GLI3R (lanes 7 and 8), similarly to endogenous GLI3 (lanes 1–4).

We also investigated endogenous GLI2 protein levels by immunoblotting analysis (Fig. 4A, middle panel). The expression of EGFP–GLI3(1–667) in control C3H10T1/2 cells substantially reduced GLI2 levels (compare lanes 3 and 4 with lanes 1 and 2). On the other hand, the expression of EGFP–GLI3(WT) in cGLI3R cells did not apparently increase GLI2 levels (compare lanes 7 and 8 with lanes 5 and 6). The GLI2 protein level data were largely consistent with the observed *Gli2* transcript levels (see below; Fig. 4F).

These cells were further analyzed for their ability to differentiate into ALP-positive cells. The differentiation of C3H10T1/2 cells into ALP-positive cells under SAG stimulation was substantially suppressed by the exogenous expression of EGFP–GLI3(1–667) (Fig. 4B, compare column 2 with column 1). On the other hand, suppression of the differentiation of cGLI3R cells into ALP-positive cells upon SAG treatment was not reversed by the exogenous expression of EGFP–GLI3(WT) (Fig. 4B, compare column 4 with column 3), even though a substantial level of the EGFP–GLI3FL protein was detected (Fig. 4A, top panel, lanes 7 and 8).

The ALP-staining data were confirmed by RT-qPCR analysis of the *Alpl* transcript. As shown in Fig. 4C, the *Alpl* level upon SAG treatment in C3H10T1/2 cells was significantly decreased by EGFP–GLI3(1–667) expression. Thus, the RT-qPCR data are largely consistent with the ALP staining data (Fig. 4B) and indicate that exogenous GLI3R can suppress *Alpl* expression in the presence of endogenous GLI3. By contrast, even under SAG-stimulation conditions, the *Alpl* transcript was not detectable in cGLI3R cells exogenously expressing EGFP–GLI3(WT). As a substantial fraction of EGFP–GLI3(WT) was processed into EGFP–GLI3R (Fig. 4A, top panel, lanes 7 and 8), it is possible that the activated form of EGFP–GLI3 was unable to overcome the repressor forms (endogenous GLI3R and EGFP–GLI3R).

Trends in the expression levels of *Gli1*, *Ptch1* and *Gli2* (Fig. 4D–F) were generally consistent with the trend in *Alpl* level (Fig. 4C). Namely, *Gli1*, *Ptch1* and *Gli2* levels upon SAG treatment in C3H10T1/2 cells were reduced by EGFP–GLI3(1–667) expression, and the levels of none of these were increased in cGLI3R cells by the expression of EGFP–GLI3(WT). These results together make it likely that GLI3R suppresses the differentiation of C3H10T1/2 cells into ALP-positive cells by suppressing not only the expression of *Gli1* but also that of *Gli2*.

GLI1 promotes osteogenic differentiation downstream of GLI3R and GLI2

To test the above possible hierarchy regarding GLI1, GLI2 and GLI3, we investigated the effects of the exogenous expression of GLI1, GLI2 or GLI3 in *Gli2*-KO and cGLI3R cells on their differentiation into ALP-positive cells. The expression of EGFP-fused human wild-type GLI1 [GLI1(WT)], GLI2(WT) and GLI3(WT) in *Gli2*-KO and cGLI3R cells was confirmed by the immunoblotting analysis of cell lysates (Fig. 5A,B). It is interesting to note that the expression of EGFP–GLI1(WT) in cGLI3R cells caused the expression of the endogenous GLI2 protein at a low but detectable level (Fig. 5B, second panel, compare lanes 5 and 6 with lanes 3 and 4), indicating that GLI1 can, at least partially, reverse the inhibition of *Gli2* expression by GLI3R.

We next investigated the differentiation of these cells into ALP-positive cells under basal and SAG-stimulated conditions (Fig. 5C). It is noteworthy that exogenously expressed EGFP–GLI1(WT) promoted the differentiation of both *Gli2*-KO and cGLI3R cells into

ALP-positive cells under not only SAG-stimulated but also basal conditions (Fig. 5C, columns 3 and 7). The ALP staining data were confirmed by RT-qPCR analysis of the *Alpl* transcript. As shown in Fig. 5D and G, the *Alpl* transcript level under basal conditions was significantly upregulated by exogenous GLI1 expression in both *Gli2*-KO and cGLI3R cells, indicating that exogenous GLI1 enhances *Alpl* expression even in the absence of GLI2, and can overcome GLI3R-mediated suppression. Thus, it is likely that exogenous GLI1 enhances *Alpl* expression independently of Hh signaling activation. These results indirectly support the idea that endogenous GLI1 functions downstream of GLI2 and GLI3R. In addition, exogenous GLI1 expression significantly increased endogenous *Gli1* expression in the basal state, in both *Gli2*-KO and cGLI3R cells (Fig. 5E,H). Furthermore, consistent with the results of GLI2 protein levels (Fig. 5B, second panel, lanes 5 and 6), *Gli2* transcript levels were also significantly increased by the expression of exogenous GLI1 (Fig. 5J). These results suggest the positive feedback of GLI1 on the expression of *Gli1* itself and *Gli2*.

On the other hand, exogenous GLI1 expression significantly increased *Ptch1* expression in *Gli2*-KO cells (Fig. 5F), but marginally in cGLI3R cells (Fig. 5I), suggesting that *Ptch1* expression might be regulated downstream of GLI2 and GLI3 and through a different pathway from that involving *Gli1*.

We then investigated whether knockdown of *Gli1* can reverse the constitutive differentiation of *Gli3*-KO cells into ALP-positive cells. Two different siRNAs for *Gli1* substantially suppressed not only the SAG-induced differentiation of control C3H10T1/2 cells into ALP-positive cells, but also the constitutive and SAG-induced differentiation of *Gli3*-KO cells into ALP-positive cells (Fig. 6A). The ALP staining data were confirmed by RT-qPCR. As shown in Fig. 6B, *Gli1* knockdown significantly decreased the SAG-induced *Alpl* level in control C3H10T1/2 cells, as well as the basal and SAG-induced *Alpl* levels in *Gli3*-KO cells. The data in Fig. 6C confirmed the effective knockdown of the *Gli1* transcript. *Ptch1* transcript levels were also decreased by *Gli1* knockdown (Fig. 6D). SAG-induced *Gli2* transcript levels in *Gli3*-KO cells were slightly decreased by *Gli1* knockdown (Fig. 6E). These results indicate that the derepression of osteogenic differentiation in the absence of GLI3 or GLI3R can be eliminated by *Gli1* knockdown.

Based on the data shown in Figs 5 and 6, we conclude that GLI1 promotes osteogenic differentiation by positive and negative regulation of its expression by GLI2 and GLI3R, respectively (Fig. 6F). In addition, GLI1 can upregulate *Gli2*, and probably also *Gli1* itself, thereby constituting a GLI1–GLI2 positive feedback loop (Fig. 6F).

DISCUSSION

Although Hh signaling is crucial for bone formation, the coordinated roles of GLI1, GLI2 and GLI3 in osteogenic differentiation were not fully understood at the cellular level. In this study, we established *Gli2*-KO, *Gli3*-KO and constitutively GLI3R-producing (cGLI3R) C3H10T1/2 cells, and analyzed their phenotypes. We then expressed GLI1, GLI2 and GLI3 constructs in these cells and knocked down *Gli1* in *Gli3*-KO cells. The data we have presented here demonstrate that GLI1 regulates osteogenic differentiation under the countervailing control by GLI2 and GLI3R.

Previous studies using mouse models, such as *Gli2*-KO, *Gli3*-KO and GLI3R-expressing mice, have suggested roles for GLI2 and GLI3R in skeletal development, although most studies have not clearly indicated whether the abnormal phenotypes are due to defects in intramembranous or endochondral bone formation. For example, *Gli2*-KO mice show craniofacial and limb abnormalities,

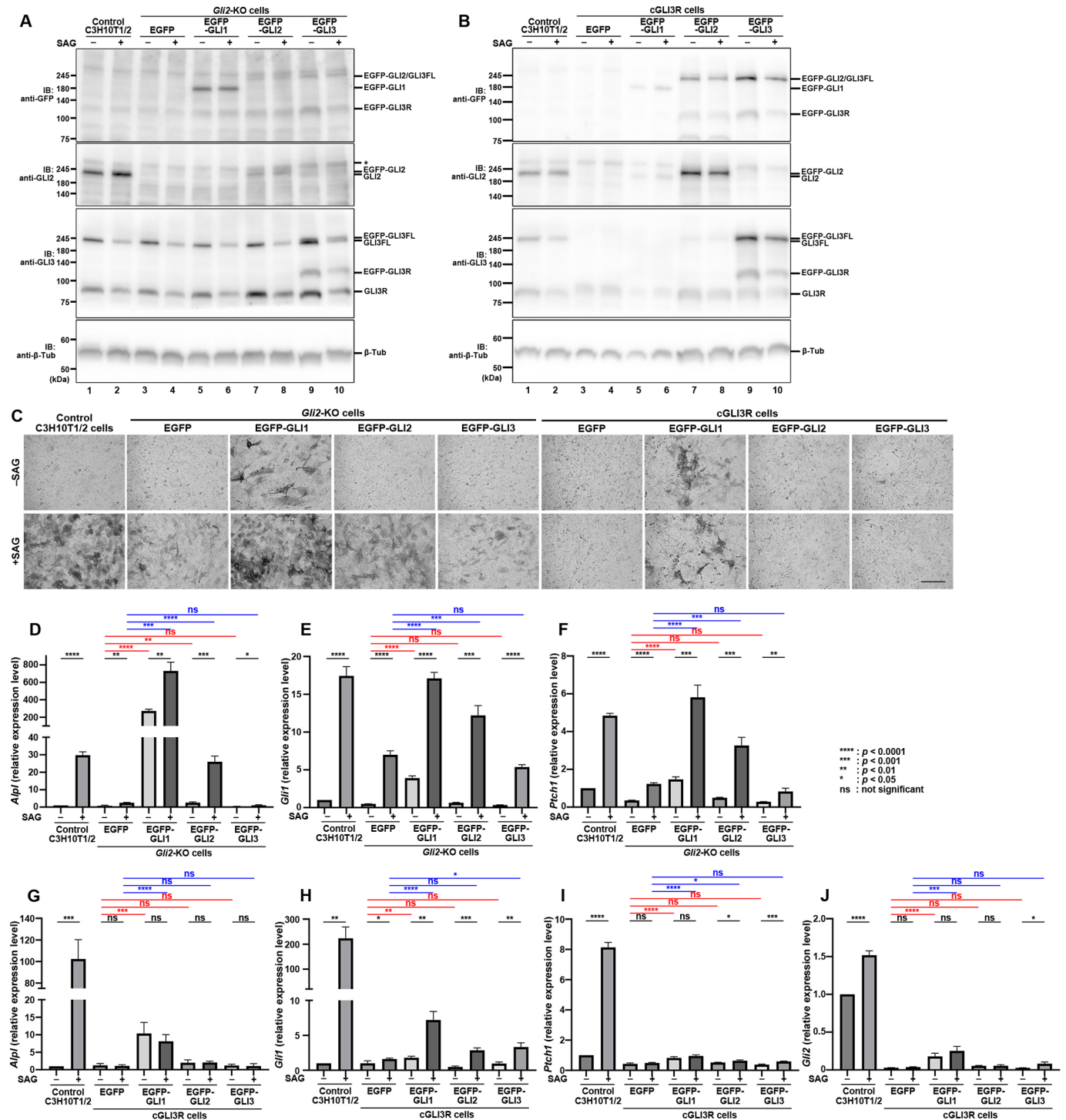


Fig. 5. Exogenously expressed GLI1 causes osteogenic differentiation and endogenous *Gli1* expression independently of GLI2 and GLI3R. Control C3H10T1/2 cells, or *Gli2*-KO or cGLI3R cells stably expressing EGFP or EGFP-fused GLI1, GLI2 or GLI3 were incubated in the absence (–SAG) or presence (+SAG) of 10 nM SAG for 7 days. (A,B) Lysates prepared from the cells were subjected to SDS-PAGE and immunoblotting (IB) analysis using an anti-GFP, anti-GLI2, anti-GLI3 or anti-β-tubulin (β-Tub) antibody. The asterisk indicates the position of nonspecific bands. Blots shown are representative of a single experiment. (C) The cells were stained for ALP activity. Scale bar: 250 μm. Images shown are representative of a single experiment. (D–J) RNA extracted from the cells was subjected to RT-qPCR for the *Alpl* (D,G), *Gli1* (E,H), *Pch1* (F,I) or *Gli2* (J) transcripts. Data are shown as the mean±s.d. of triplicates. Statistical significances among multiple groups and those between –SAG and +SAG were calculated using one-way ANOVA followed by Dunnett's multiple comparison test and two-tailed unpaired Student's *t*-test, respectively.

suggesting roles of GLI2 in both intramembranous and endochondral bone formation (Mo et al., 1997). Another study has reported that the GLI2 activator function is sufficient to induce osteoblastic differentiation in the perichondrium during endochondral

bone formation (Kesper et al., 2010). On the other hand, *Gli3*-null mice demonstrate craniosynostosis due to enhanced osteoblastic differentiation, suggesting derepression of intramembranous bone formation in the absence of *Gli3* (Rice et al., 2010). A recent study

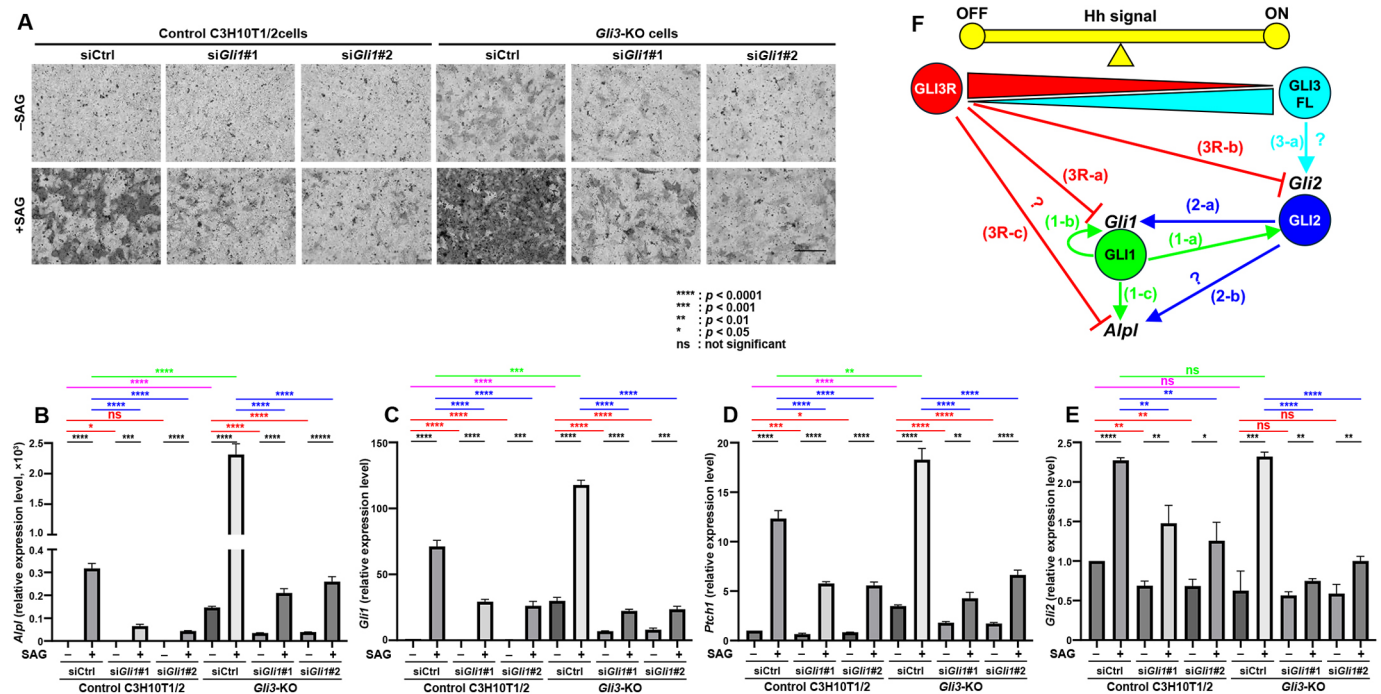


Fig. 6. *Gli1* knockdown suppresses osteogenic differentiation of control C3H10T1/2 and *Gli3*-KO cells. Control C3H10T1/2 cells or *Gli3*-KO cells were treated with control siRNA (siCtrl) or siRNAs for *Gli1* (si*Gli1*#1 or si*Gli1*#2) and were incubated in the absence (–SAG) or presence (+SAG) of 200 nM SAG for 3 days. (A) The cells were stained for ALP activity. Scale bar: 250 μ m. Images shown are representative of two experiments. (B–E) RNA extracted from the cells was subjected to RT-qPCR for the *Alpl* (B), *Gli1* (C), *Pthch1* (D) or *Gli2* (E) transcripts. Data are shown as the mean $\pm$ s.d. of triplicates. Statistical significances among multiple groups and those between –SAG and +SAG were calculated using one-way ANOVA followed by Dunnett's multiple comparison test and two-tailed unpaired Student's *t*-test, respectively. (F) Schematic model of the regulation of *Gli1*, *Gli2* and *Alpl* expression by GLI1, GLI2 and GLI3. This model is explained in the text (see Discussion).

analyzing phenotypes of conditional *Gpr161*-KO mice with additional *Gli2*-KO, *Gli3*-KO or GLI3R knock-in has proposed tissue-specific regulation of intramembranous bone formation under the control of GLI2 and GLI3R: the GLI3R level determines intramembranous bone formation in the humerus and in the medial parietal bone of the calvarium; by contrast, the balance between GLI2 and GLI3R regulates intramembranous bone formation in most of the ulna and radius and in most of the calvarium (except for the medial parietal bone) (Hwang et al., 2023). This is in line with the counterregulatory roles of GLI2 and GLI3R in osteogenic differentiation of C3H10T1/2 cells confirmed in the present study.

Based on the data presented in the present study, together with previous studies, we propose the following model of the regulation of *Gli1*, *Gli2* and *Alpl* expression by GLI1, GLI2 and GLI3 (Fig. 6F). GLI3R, which results from GLI3FL processing requiring PKA-mediated phosphorylation, represses the expression of not only *Gli1* (Fig. 6F, 3R-a) but also *Gli2* (Fig. 6F, 3R-b), and GLI2 upregulates *Gli1* (Fig. 6F, 2-a). GLI1 can upregulate the expression of *Gli2* (Fig. 6F, 1-a) as well as *Gli1* itself (Fig. 6F, 1-b), indicating the presence of a GLI1–GLI2 positive feedback loop. Finally, GLI1 enhances the expression of genes required for osteogenic differentiation, including *Alpl* (Fig. 6F, 1-c). It is a subject of future study whether GLI3R and GLI2 can directly regulate *Alpl* expression (Fig. 6F, 3R-c and 2-b).

Gli3-KO cells underwent differentiation into osteoblasts even in the absence of Hh pathway stimulation (Fig. 1G,H), indicating basal derepression of the signaling in the absence of GLI3. Namely, endogenous GLI3 predominantly functions as a repressor of osteogenic differentiation. In clear contrast, *Gli2*-KO cells were severely impaired in osteogenic differentiation, even when the Hh pathway was stimulated by SAG treatment (Fig. 1G,H), indicating

that endogenous GLI2 functions as a Hh-dependent activator of osteogenic differentiation. On the other hand, cGLI3R cells cannot undergo differentiation at all (Fig. 1G,H), probably owing to the repression of both *Gli1* and *Gli2* genes (Fig. 1I,K).

Whether the *Gli2*-KO, *Gli3*-KO and cGLI3R cells differentiate into ALP-positive cells or not was highly correlated with their *Gli1* expression levels (compare Fig. 1G and H with I), suggesting that GLI1 regulates osteogenic differentiation at the most downstream step of the Hh pathway. This is consistent with previous studies showing that *Gli1*-positive cells in *Gli1* reporter mice have characteristics of skeletal stem cells with osteogenic potential (Jin et al., 2024; Zhao et al., 2015). The counteracting roles of GLI2 and GLI3 in the regulation of osteogenic differentiation via *Gli1* expression was supported by data based on exogenous GLI1 expression in *Gli2*-KO and cGLI3R cells, and *Gli1* knockdown in *Gli3*-KO cells. Namely, forced GLI1 expression restored osteogenic differentiation in the absence of GLI2 or the constant presence of GLI3R (Fig. 5), and *Gli1* knockdown blocked the constitutive differentiation of *Gli3*-KO cells (Fig. 6). An apparent discrepancy is that when *Gli2*-KO cells were stimulated with SAG, *Alpl* and *Gli1* levels were significantly upregulated (Fig. 1G–I); as previously proposed (Buttitta et al., 2003; Mo et al., 1997; Motoyama et al., 2003), under artificial conditions of the absence of endogenous GLI2, GLI3 might be able to act as an activator to induce *Gli1* expression.

It is also noteworthy that the expression of both *Gli1* and *Gli2* was virtually undetectable in cGLI3R cells (Fig. 1I,K). Thus, GLI3R is likely to repress *Gli2* expression, in addition to repressing *Gli1* expression. Furthermore, we showed that GLI2 upregulates *Gli1* expression, and GLI1 upregulates the expression of *Gli1* itself and *Gli2*. This is compatible with previous studies suggesting the

presence of a GLI1–GLI2 positive feedback loop (Regl et al., 2002; Taylor et al., 2019).

EGFP-fused GLI3(WT), but not GLI3(6SA) or GLI3(6SD), underwent processing to the GLI3R form (Fig. 3A, top panel, lanes 5–10), in agreement with a previous study using knock-in mice (Wang et al., 2007). EGFP-fused GLI3(WT) and GLI3(1–667), but not GLI3(6SA) or GLI3(6SD), eliminated the constitutive differentiation of *Gli3*-KO cells (Fig. 3). These results support the notion that GLI3 predominantly functions as an inhibitor of Hh signaling after its processing to a GLI3R form in the basal state.

On the other hand, when EGFP-fused GLI2(6SA) or GLI2(6SD) was expressed in *Gli2*-KO cells, *Alpl* and *Gli1* levels under basal conditions were significantly increased compared with those in *Gli2*-KO cells expressing EGFP–GLI2(WT) (Fig. 2C,D). These results suggest that the PKA-mediated phosphorylation of GLI2 in the basal state suppresses its activator function in some way without its conversion to a smaller, repressor form (see Fig. 1B, top panel, lanes 1 and 2; and Fig. 2A, top panel, lanes 5 and 6). A previous study using the transfection of GLI2 constructs into HEK293 cells has suggested that the PKA-mediated phosphorylation of GLI2 is required for its polyubiquitylation and subsequent degradation by proteasomes (Pan et al., 2006). However, the EGFP-fused GLI2(WT) protein under basal conditions did not appear to be degraded more extensively than nonphosphorylatable GLI2(6SA) and GLI2(6SD) (Fig. 2A, top panel, lanes 5–10), even though basal *Alpl* and *Gli1* transcript levels were significantly lower in cells expressing EGFP–GLI2(WT) than in those expressing EGFP-fused GLI2(6SA) or GLI2(6SD) (Fig. 2C,D). Thus, how PKA-mediated phosphorylation of GLI2 suppresses its activator function is an issue to be addressed in the future.

Finally, it is interesting to note that treatment of C3H10T1/2 cells with ascorbic acid and β -glycerophosphate might promote osteogenic differentiation by a mechanism different from that involving Hh signaling (see Fig. S2). In other words, osteogenic differentiation observed by culturing MSCs in the conventionally used osteogenic medium containing ascorbic acid and β -glycerophosphate might not reflect differentiation mediated by Hh signaling. The mechanism by which such treatment promotes osteogenic differentiation is an issue for future studies.

MATERIALS AND METHODS

Antibodies, plasmids and oligodeoxyribonucleotides

Antibodies, chemicals and plasmids used in this study are listed in Table S1. Oligodeoxyribonucleotides used in this study are listed in Table S2.

Cell lines

C3H10T1/2 clone 8 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition (JCRB9080), and cultured in Basal Medium Eagle (BME; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 2 mM L-glutamine at 37°C in 5% CO₂. For osteogenic differentiation, the cells were cultured in α -modified MEM (α -MEM; Nacalai Tesque) containing 10% FBS supplemented either with SAG (10 nM for 3 or 7 days) or with ascorbic acid and β -glycerophosphate (50 μ g/ml and 10 mM, respectively, for 7 days). HEK293T cells were obtained from RIKEN BioResource Research Center (RBC2202) and cultured in DMEM with high glucose (Nacalai Tesque) supplemented with 5% FBS.

Establishment of KO cell lines

Disruption of the *Gli2* or *Gli3* genes in C3H10T1/2 cells using the CRISPR/Cas9 system by homology-independent DNA repair was performed as previously described in detail (Katoh et al., 2017), with some modifications (Fujisawa et al., 2021; Okazaki et al., 2020; Qiu et al., 2021). Single-guide RNA (sgRNA) sequences targeting mouse *Gli2* (Target #1) or *Gli3* (Target #2

or Target #3) (Table S2; also see Fig. 1A) were designed using CRISPOR (Haeussler et al., 2016). Double-stranded oligonucleotides for the target sequences were inserted into the all-in-one sgRNA expression vector pHiFiCas9-2 $\times$ sgRNA (Fujisawa et al., 2021). C3H10T1/2 cells were seeded onto a 12-well plate to $\sim 2.0 \times 10^4$ cells per well, and on the next day they were transfected with the sgRNA vector (1 μ g) and the donor knock-in vector, pDonor-tBFP-NLS-Neo (universal) (0.25 μ g), using X-tremeGENE9 reagent. After selection in the presence of G418 (600 μ g/ml), the cells were sorted using the SH800S cell sorter (SONY) at the Medical Research Support Center of Kyoto University. To confirm the disruption of the targeted gene in the isolated cell lines, genomic DNA extracted from the isolated cells were subjected to PCR using GoTaq Master Mixes and three sets of primers (Table S2) to distinguish the following three integration states of the donor knock-in vector: forward integration, reverse integration and no integration with a small indel (see Katoh et al., 2017). Direct sequencing of the genomic PCR products was performed to confirm the disruption of alleles of the *Gli2* or *Gli3* gene (Fig. S1).

Preparation of lentiviral vectors and establishment of stable cell lines

cDNAs for human GLI1, GLI2 and GLI3 were obtained from Addgene (#84922, #84920, and #65435, respectively). Lentiviral vectors for the stable expression of the GLI1, GLI2 and GLI3 constructs were prepared as previously described (Hamada et al., 2018; Takahashi et al., 2012). Briefly, HEK293T cells were cotransfected with EGFP-fused GLI1, GLI2 and GLI3 constructs (see Table S1) in pRRLsinPPT (Thomas et al., 2009), and the packaging plasmids (pRSV-REV, pMD2.g and pMDLg/pRRE) (Thomas et al., 2009) using Polyethylenimine Max. Culture medium was replaced 8 h after transfection and was collected at 24, 36 and 48 h after transfection. The medium containing viral particles was passed through a 0.45 μ m filter and centrifuged at 32,000 *g* at 4°C for 4 h to precipitate the viral particles, which were resuspended in BME supplemented with 0.5% FBS and 2 mM L-glutamine, and stored at -80°C until use. The pRRLsinPPT, pRSV-REV, pMD2.g and pMDLg/pRRE plasmids were provided by Peter McPherson (McGill University, Montreal, Canada).

Treatment of cells with siRNAs for *Gli1*

Cells were grown on a 6-well plate to at least 70% confluence before transfection. Transient knockdown was performed by the transfection of cells with siRNAs listed in Table S2 (final concentration of 20 nM) using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) for 24 h according to the manufacturer's instructions.

Immunofluorescence analysis

To induce ciliogenesis, cells were grown on coverslips to 100% confluence, and starved for 24 h in BME supplemented with 0.5% FBS and 2 mM L-glutamine. Immunofluorescence analysis was performed as described previously (Tasaki et al., 2023; Zhou et al., 2022b). For immunostaining of endogenous proteins, cells were fixed and permeabilized under the following conditions: 3% paraformaldehyde for 5 min at room temperature, followed by methanol for 5 min at -20°C for ARL13B, GLI2 and GLI3. The fixed and permeabilized cells were washed three times with phosphate-buffered saline, blocked with 10% FBS, and incubated sequentially with primary and secondary antibodies diluted in 5% FBS. The immunostained cells were observed using an Axio Observer microscope using a 63 $\times$ /1.4 objective lens (Carl Zeiss).

For quantitative analysis, all images were acquired under the same setting and were analyzed using ZEN 3.1 software (Carl Zeiss). A region of interest (ROI) was constructed along the signal of ARL13B within cilia using a drawing tool, and the fluorescence intensity in the ROI was quantified. To correct for local background intensity, the ROI was duplicated and set to a nearby region. Statistical analyses were performed using GraphPad Prism 10 software (version 10.1.2; GraphPad Software, Inc.).

SDS-PAGE and immunoblotting analysis

To induce ciliogenesis, cells were grown on six-well plates to 100% confluence, and starved for 24 h in BME supplemented with 0.5% FBS and 2 mM L-glutamine. The culture medium was changed to the above medium containing 200 nM SAG, and the cells were incubated for a further 6 h. The cells were lysed in 100 μ l of cell lysis buffer [50 mM Tris-HCl (pH 7.4),

150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA] containing EDTA-free protease inhibitor cocktail (Nacalai Tesque). After 20 min on ice, the cell lysates were centrifuged at 16,100 *g* for 15 min at 4°C in a microcentrifuge. The supernatants were boiled in SDS-PAGE sample buffer and separated by SDS-PAGE using gels prepared with TGX FastCast Acrylamide Solution Kit 10% (Bio-Rad). The separated proteins were electroblotted onto an Immobilon-P membrane (Merck-Millipore), and the membrane was then subjected to immunoblotting analysis using an anti-GLI2, anti-GLI3, anti-GFP or anti- β -tubulin antibody followed by a peroxidase-conjugated secondary antibody. Protein bands were detected using a Chemi-Lumi One L kit (Nacalai Tesque), and images were captured using ImageQuant 800 (Cytiva). The intensity of the bands was measured using ImageJ software (National Institutes of Health). Full blot images are shown in Fig. S6.

ALP staining

Cells were incubated in the absence (–SAG) or presence (+SAG) of 10 nM SAG for 7 days. The cells were then fixed in 3% paraformaldehyde for 15 min at room temperature, washed with phosphate-buffered saline and stained with Black Substrate Kit Alkaline Phosphatase (Vector Laboratories) for 30 min at room temperature. The stained cells were washed with 100 mM Tris-HCl (pH 9.5) for 5 min and observed using an all-in-one-type fluorescence microscope EVOS M5000 (Thermo Fisher Scientific) using a 10×/0.40 objective lens.

RT-qPCR

Total RNA was isolated from cultured cells using an RNeasy mini kit (QIAGEN). Reverse transcription was performed using ReverTra Ace qPCR RT Master Mix (TOYOBO) followed by RT-qPCR using the StepOnePlus system (Thermo Fisher Scientific). THUNDERBIRD SYBR qPCR Mix (TOYOBO) containing 0.3 μ M of a specific primer set corresponding to each gene (Table S2) was used in the reactions. Data were quantified using the comparative CT method (delta CT method; Schmittgen and Livak, 2008) to estimate the mRNA copy number relative to that of mouse 18S rRNA, which was used as internal standard.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.T., Y.I., S.Y., H.-W.S., Y.K., K.N.; Formal analysis: Y.T., Y.I.; Funding acquisition: Y.I., Y.K., K.N.; Investigation: Y.T., Y.I.; Resources: S.Y.; Supervision: H.-W.S., Y.K.; Visualization: Y.T., Y.I.; Writing – original draft: K.N.; Writing – review & editing: Y.T., Y.I., S.Y., H.-W.S., Y.K., K.N.

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Data availability

All relevant data can be found within the article and its [supplementary information](#).

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