Gli1 Protein Participates in Hedgehog-mediated Specification of Osteoblast Lineage during Endochondral Ossification*

Hironori Hojo, Shinsuke Ohba, Fumiko Yano, Taku Saito, Toshiyuki Ikeda, Keiji Nakajima, Yusuke Komiyama, Naomi Nakagata, Kentaro Suzuki, Tsuyoshi Takato, Hiroshi Kawaguchi, and Ung-il Chung

From the Center for Disease Biology and Integrative Medicine and Department of Sensory and Motor System Medicine, The University of Tokyo, 113-0033 and the Center for Animal Resources and Development (CARD), Kumamoto University, Kumamoto 860-0811, Japan.

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Background: Gli1 was thought to be dispensable for normal embryogenesis.

Results: Gli1 physiologically acts as an osteogenesis-related Gli activator and mediates the functions of Gli2 and Gli3 in osteogenesis.

Conclusion: Gli1 collectively functions with Gli2 and Gli3 in osteogenesis.

Significance: This study provides insight into the elaborate molecular framework of a context-dependent divergence of functions of Hedgehog-Gli signaling in development.

With regard to Hedgehog signaling in mammalian development, the majority of research has focused on Gli2 and Gli3 rather than Gli1. This is because Gli1−/− mice do not show any gross abnormalities in adulthood, and no detailed analyses of fetal Gli1−/− mice are available. In this study, we investigated the physiological role of Gli1 in osteogenesis. Histological analyses revealed that bone formation was impaired in Gli1−/− fetuses compared with WT fetuses. Gli1−/− perichondrial cells expressed neither runt-related transcription factor 2 (Runx2) nor osterix, master regulators of osteogenesis, in contrast to WT cells. In vitro analyses showed that overexpression of Gli1 up-regulated early osteogenesis-related genes in both WT and Runx2−/− perichondrial cells, and Gli1 activated transcription of those genes via its association with their 5′-regulatory regions, underlying the function of Gli1 in the perichondrium. Moreover, Gli1−/−;Gli2−/− mice showed more severe phenotypes of impaired bone formation than either Gli1−/− or Gli2−/− mice, and osteoblast differentiation was impaired in Gli1−/−;Gli3−/− perichondrial cells compared with Gli3−/− cells in vitro. These data suggest that Gli1 itself can induce early osteoblast differentiation, at least to some extent, in a Runx2-independent manner. It also plays a redundant role with Gli2 and is involved in the repressor function of Gli3 in osteogenesis. On the basis of these findings, we propose that upon Hedgehog input, Gli1 functions collectively with Gli2 and Gli3 in osteogenesis.

Hedgehog (Hh) signaling is a highly conserved pathway known to play essential roles in various developmental processes. Gli1, Gli2, and Gli3 are thought to mediate transcriptional responses to Hh input in a context-dependent manner (1). Gli2 is suggested to function primarily as a transcriptional activator, and Gli3 as a transcriptional repressor (1). Gli2 and Gli3 have been demonstrated to play major roles in Hh signaling in mouse development (2, 3). In contrast, Gli1, a transcriptional activator, is thought to be dispensable for normal development (4, 5). However, several lines of evidence have indicated that Gli1 plays an important role in pathological conditions (3, 6).

Endochondral ossification is among the notable events regulated by Indian hedgehog (Ihh) (7). During this process, perichondrial cells adjacent to the mineralized hypertrophic chondrocytes turn into osteoblast precursors expressing alkaline phosphatase (Alp), bone sialoprotein (Bsp), and the type I line phosphatase; Bsp, bone sialoprotein; Col1a1, type I collagen α1 chain; Oc, osteocalcin; Runx2, Runt-related transcription factor 2; Oss, osterix; Col2a1, type II collagen α1 chain; E, embryonic day; Col10a1, type X collagen α1 chain.

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† Supported by a Japan Society for the Promotion of Research Fellowship. To whom correspondence may be addressed: Center for Disease Biology and Integrative Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: 81-3-5841-1427; Fax: 81-3-5841-1428; E-mail: hojo@bmw.t.u-tokyo.ac.jp.

‡ To whom correspondence may be addressed: Center for Disease Biology and Integrative Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: 81-3-5841-1427; Fax: 81-3-5841-1428; E-mail: soba@bmw.t.u-tokyo.ac.jp.
**EXPERIMENTAL PROCEDURES**

**Animals**—Ihh
\(^{tm1Amc}\) (Ihh\(^{+/-}\)) mice were obtained from The Jackson Laboratory, WT C57BL/6j mice were from Charles River Japan, and Gli1\(^{+/-}\) and Gli2\(^{+/-}\) mice were generated as described previously (4, 15). Runx2\(^{+/-}\) mice were a gift from T. Komori (Nagasaki University, Nagasaki, Japan) (9). All experiments were performed in accordance with the protocol approved by the Animal Care and Use Committee of The University of Tokyo.

**Reagents and Vectors—**SAG (Smoothened agonist) was purchased from Calbiochem, and pEGFP-C1 was from Clontech. Plasmids expressing Gli1 were generated as described previously (12). The shRNA expression vector for Gli1 was constructed using the pGENEmU6 vector (iGENE Therapeutics). The target sequences were as follows: Gli1-1, AACTCCACAGGCACACAGGAT; and Gli1-2, GCCTTACATGTGTGAGCTGGGAAGCAAGCGTCACT (forward) and CTGTCTGGGTGCCAGAG (reverse); Gli2, GCCTTACATGTGTGAGCTGGGAAGCAAGCGTCACT (forward) and CTGTCTGGGTGCCAGAG (reverse); and Gli1, GCACCCACATCAAAGTGACGAC (forward) and CCGCAGCACAACCGACCACTC (reverse).

**Cell Culture**—C3H10T1/2 cells were obtained from the RIKEN Cell Bank. Perichondrial cells were isolated as described previously (16). For immunohistochemistry of the fetus, Alexa Fluor 488 (1:500 dilution; A-11034, Invitrogen) was used for detection. Alp staining was performed as described previously (16). ALP staining was performed as described previously (16). Applied Science). ALP staining was performed as described previously (16). The shRNA expression vector for Gli1 was constructed using the pGENEmU6 vector (iGENE Therapeutics). The target sequences were as follows: Gli1-1, AACTCCACAGGCACACAGGAT; and Gli1-2, GCCTTACATGTGTGAGCTGGGAAGCAAGCGTCACT (forward) and CTGTCTGGGTGCCAGAG (reverse); Gli2, GCCTTACATGTGTGAGCTGGGAAGCAAGCGTCACT (forward) and CTGTCTGGGTGCCAGAG (reverse); and Gli1, GCACCCACATCAAAGTGACGAC (forward) and CCGCAGCACAACCGACCACTC (reverse).

**Histology**—The procedures for von Kossa staining and in situ hybridization have been described previously (18). Immunohistochemistry was performed using anti-Runx2 (1:50 dilution; sc-10758, Santa Cruz Biotechnology, Inc.) and anti-osterix (1:5000 dilution; ab22552, Abcam) antibodies as described previously (16). For immunohistochemistry of the Gli1\(^{+/-}\) fetus, frozen sections were prepared at 10 \(\mu\)m. Alexa Fluor 488 (1:500 dilution; A-11034, Invitrogen) was used for detection.

**Microscopy**—Images were taken using an Axio Imager A1 or Axiovert 200 microscope (Carl Zeiss) and processed using AxioVision (Carl Zeiss).

**Luciferase Assay**—Cells were plated onto 24-well plates and transfected with 0.4 \(\mu\)g of DNA mixture containing the test reporter plasmids, control reporter plasmids encoding Renilla luciferase, and effector plasmids. A Dual-Luciferase assay was performed as described previously (12).

**EMSA**—Nuclear extracts were obtained as described previously (19). Digoxygenin-labeled probes were constructed using a DIG gel shift kit (Roche Applied Science). A total of 1 \(\mu\)g of nuclear extracts of GFP- and Gli1-transfected C3H10T1/2 cells was used. The binding mixture contained 50 mm KCl, 25 mm HEPES-KOH (pH 7.9), 5 mm MgCl\(_2\), 10 mm ZnSO\(_4\), 1 mm DTT, 12% glycerol, 0.1% Nonidet P-40, 0.25 \(\mu\)g of poly(dI-dC), 0.1 \(\mu\)g of poly-L-lysine, and 30 fmol of the digoxigenin-labeled probe. The binding reaction was performed for 30 min at room temperature, and a 50-fold molar excess of unlabeled probes was used for the competition. When antibodies were used, nuclear extracts were incubated with 1 \(\mu\)g of anti-GLI1 antibody (sc-20687X, Santa Cruz Biotechnology, Inc.) or non-immune IgG (sc-2027, Santa Cruz Biotechnology, Inc.) for 30 min at room temperature prior to the binding reaction. Electrophoresis was performed with a 6% Tris borate/EDTA gel (Invitrogen) in 0.25\(\times\) Tris borate/EDTA at 150 V for 60 min, and samples were subjected to blotting on nylon membranes and detection for digoxigenin-labeled probes.

Oligonucleotide probes containing the Gli-G/C-containing region in the human ALP region and the Gli motif-containing region in the human BSP region were used, and their mutations were as follows: Gli-G/C-containing region in the WT human ALP region, 5'-GGGCTCGGGCCCGGAAGCGGGCGGG-3'; Gli-G/C-containing region in the human ALP region mutant, 5'-GGGCTCGGGCCCGGAAGCGGGCGGG-3'; Gli motif-containing region in the WT human BSP region, 5'-TGACAGTGATTGGCTGTGGGAAGGC-3'; and Gli motif-containing region in the human BSP region mutant, 5'-TGACAGTGATTGCAAGGCGGC-3'.

**ChiP—**ChiP was performed with a OneDay ChIP kit (Diagenode) using 5 \(\mu\)g of anti-GLI1 antibody, anti-GFP antibody (ab290, Abcam), non-immune IgG, and anti-acetyl-histone H3 antibody (06-599, Upstate) following the manufacturer's instructions. To shear genomic DNA, a shearing ChiP kit (Diagenode) was used according to the manufacturer's instructions. PCR was performed after purification of DNA. The primer sequences were as follows: -220 to -21 in the Alp region, CAGGATGAGCCGGCAAGGAG (forward) and ATGAAAGGAGCTGGGCGG (reverse); -1000 to -801 in the Alp region, ACCCCCTCTTTCATCAAAATGTT (forward) and CTACACTTTTCCCTTGTTGTGCTTCTG (reverse); -240 to -41 in the Bsp region, GAACCTCTAC- TACCATCTTCTCTCCACCT (forward) and CCTTCTTCCATCTT- TGGCG (reverse); and -2000 to -1801 in the Bsp region, AGGGCTAATCTATGCTTTGAGATGGC (forward) and TGTACAGAGACCACCCCGTGAGAAATGTG (reverse).

**Statistical Analysis**—The means of groups were compared by analysis of variance, and the significance of differences was determined by post hoc testing using Tukey's method.

**RESULTS**

**Gli1 Contributes to Hh Signaling-mediated Osteoblast Differentiation**—To examine the contribution of Gli1 to Hh signaling-mediated osteoblast differentiation compared with
Gli1 and Specification of Osteoblast Lineage

FIGURE 1. Involvement of Gli1 in Hh-mediated osteoblast differentiation. A, ALP staining and Alp mRNA expression determined by real-time RT-PCR in perichondrial cells from WT, Gli1−/−, Gli2−/−, and Gli3−/− mouse embryos (E17.5). Cells were treated with SAG for 7 days for ALP staining and for 3 days for real-time RT-PCR analysis. *, p < 0.05. B, mRNA expression of osteoblast marker genes determined by real-time RT-PCR in C3H10T1/2 cells. Cells were transfected with the GFP (1 μg) or Gli1 (0.1 or 1 μg) plasmid and cultured for 48 h. C, mRNA expression of osteoblast marker genes determined by real-time RT-PCR in C3H10T1/2 cells. After being transfected with shRNA for GFP or Gli1, the cells were cultured for 12 h, followed by exposure to SAG (1 μg) for 48 h. shGli1, Gli1 shRNA. *, p < 0.05. For A–C, data are means ± S.D. of triplicate wells, and representative data of independent experiments are shown.

Gli2 and Gli3, we examined the induction of osteoblast differentiation by SAG, a Smoothened agonist, in perichondrial cells isolated from Gli knock-out or WT mice. SAG-induced osteoblast differentiation was impaired in Gli1−/− and Gli2−/− cells relative to that in WT cells as shown by a decrease in ALP staining intensity and Alp mRNA expression, whereas it was enhanced in Gli3−/− cells (Fig. 1A). In addition, overexpression of Gli1 alone induced mRNA expression of Alp and Bsp, early and middle markers of osteoblast differentiation. Overexpression of Gli1 alone did not affect Oc mRNA (Fig. 1B), a later maker in the C3H10T1/2 cell line, which has been reported to be able to differentiate into osteoblastic cells upon Hh signaling (20). shRNA for Gli1 inhibited the SAG-induced expression of Gli1, Alp, and Bsp in C3H10T1/2 cells (Fig. 1C). These data suggest that in addition to Gli2 and Gli3, Gli1 contributes to Hh-mediated osteoblast differentiation, mainly in its early phase.

Gli1 Is Involved in Hh Signaling-mediated Specification of Osteoblast Lineage—Although in vitro data have suggested that Gli1 contributes to osteoblast differentiation, Gli1−/− mice are viable and appear normal in adulthood (4), and no detailed analyses in fetal Gli1−/− mice are available. Therefore, we investigated the physiological roles of Gli1 in endochondral ossification by analyzing the skeletal phenotypes of fetal Gli1−/− mice. At embryonic day (E) 15.5, the Gli1−/− perichondrium, unlike the WT perichondrium, did not express Alp. There was no difference in the expression pattern of chondrogenic markers, including Col2a1, type X collagen α1 chain (Col10a1), and Ihh, between WT and Gli1−/− mice (Fig. 2). At E17.5, no bone collar was observed in Gli1−/− mice, even though mineralized chondrocytes were already present. The Gli1−/− perichondrium showed a decreased region of Alp-expressing cells compared with the WT perichondrium, and it lacked Bsp expression (Fig. 2). Expression of Runx2 or Osx was not discernible in the Gli1−/− perichondrial cells at this stage (Fig. 2), indicating that osteogenesis in the Gli1−/− perichondral cells was delayed.

Because the expression of Runx2 and Osx is absent in the Ihh−/− perichondrium (7, 13), this suggests that the Gli1−/− fetus partially phenocopies Ihh−/− in osteogenesis. We then tested whether Gli1 overexpression could rescue the phenotype of the Ihh−/− perichondrium in vitro. Perichondrial cells of Ihh−/− embryos showed decreased mRNA expression of Alp and Bsp compared with WT cells (Fig. 3). Gli1 overexpression rescued the expression of Alp completely and that of Bsp partially (Fig. 3). These data suggest that, at least in part, Gli1 is likely to contribute to the specification of the osteoblast lineage downstream of Ihh.

Gli1 Itself, as Well as Activation of Hh Signaling, Can Induce Early Osteoblast Differentiation in Runx2-independent Manner—Our data up to this point led us to consider two possibilities for molecular mechanisms underlying the induction of
early osteoblast differentiation by the Hh-Gli1 pathway: 1) indirect action via induction of Runx2 and 2) Gli1-mediated transcriptional regulation of osteogenesis-related genes. We initially focused on the first possibility and investigated whether activation of the Hh-Gli1 pathway could induce osteoblast differentiation in Runx2/H11002/H11002 perichondrial cells in vitro. Activation of Hh signaling with SAG up-regulated mRNA expression of osteogenesis-related genes, including Alp, Bsp, and Oc, as well as Runx2, in WT cells (Fig. 4A). In Runx2−/− perichondrial cells, SAG up-regulated Alp and Bsp, but not Oc (Fig. 4A). Over-expression of Gli1 up-regulated Alp and Bsp, but not Oc, in both WT and Runx2−/− cells, and it did not alter the mRNA expression of Runx2 and Oc in WT cells (Fig. 4B). The expression levels of Alp and Bsp induced by SAG or Gli1 in Runx2−/− cells were lower than those in WT cells (Fig. 4, A and B). These data indicate that activation of the Hh-Gli1 pathway can induce some markers of the early stage of osteoblast differentiation in a Runx2-independent manner.

Gli1 Transactivates Osteogenesis-related Genes via Its Association with Their 5’-Regulatory Regions—We then verified the second possible molecular mechanism underlying the induction of early osteoblast differentiation by the Hh-Gli1 pathway. We hypothesized that Gli1 regulates the transcription of Alp and Bsp via its association with the 5’-regulatory regions of
Gli1 Functions Collectively with Gli2 and Gli3 in Osteogenesis—We next attempted to clarify how Gli1 functions in osteogenesis in association with Hh input, Gli2, and Gli3. First, we confirmed that Gli1 acted downstream of Gli2 and Gli3 in the perichondrium, as shown previously in other tissues (22). Gli2−/− perichondrial cells showed decreased induction of Gli1 expression by SAG compared with WT cells. This induction was increased in Gli3−/− cells (Fig. 7A). In contrast, Gli1−/− cells did not alter Gli2 or Gli3 mRNA expression, protein expression, or protein subcellular localization in vitro (data not shown).

Second, given that skeletal phenotypes observed in Gli1−/− metatarsals at E17.5 were partially normalized at postnatal day 0 (Fig. 7B), we hypothesized that Gli plays a redundant role in this context. Because genetic and functional redundancies between Gli1 and Gli2 have been shown in the development of the CNS and lung lobes (4, 22, 23), we analyzed the skeletal tissues of Gli1−/−;Gli2−/−;Ihh−/− mice at postnatal day 0. The bone collar was absent in Gli1−/−;Gli2−/−;Ihh−/− metatarsals and tibias and partially impaired in Gli1−/−;Gli2−/−;Ihh−/− femurs (Fig. 7B). In Gli1−/−;Gli2−/− metatarsals, perichondrial cells did not express Col1a1 or Oss. In contrast, the WT, Gli1−/−, and Gli2−/− metatarsals appeared normal in terms of the expression pattern of osteoblast marker genes (Fig. 7C). These data...
suggest that Gli1 and Gli2 play redundant roles in the specification of the osteoblast lineage in the perichondrium. Finally, regarding the interaction of Gli1 and Gli3, our data had raised the possibility that induction of osteogenesis in Gli3/−/− perichondrial cells was mediated by the expression of Gli1. We found that Gli1/−/−;Gli3/−/− perichondrial cells showed decreased expression of Alp compared with Gli3/−/− cells in vitro (Fig. 7D). These data suggest that the inhibitory actions of the Gli3 repressor on osteoblast differentiation are mediated, at least in part, by the inhibition of Gli1 expression.

**DISCUSSION**

This study had four major findings. First, in addition to Gli2 and Gli3, Gli1 is involved in the Hh signaling-mediated specification of the osteoblast lineage. Second, Gli1 induces an early stage of osteoblast differentiation, at least to some extent, in a Runx2-independent manner. Third, Gli1 promotes the transcription of osteogenesis-related genes via its binding to DNA, which possibly accounts for the activity of Gli1 in the perichondrium. Finally, Gli1 plays a redundant role with Gli2 and is involved in the repressor function of Gli3 in osteogenesis. On the basis of these findings, we propose that Gli1 functions collectively with Gli2 and Gli3 in osteogenesis, playing a pivotal role in the specification of the osteoblast lineage upon Hh input. In this context, Gli1 may specifically promote early osteoblast differentiation, at least to some extent, in a Runx2-independent manner.

Our findings shed new light on the physiological function of Gli1 in a context-dependent manner. We observed abnormalities in osteogenesis in the Gli1/−/− perichondrium, but chondrogenesis in Gli1/−/− growth plates appeared normal, even though Ihh regulates both of these processes (24, 25). These data suggest that the transient requirement of Gli1 at a certain point of differentiation appears to depend on cell types, even in skeletogenesis. In line with our findings, several groups recently reported that Gli1 affected the proliferation and differentiation of hematopoietic stem cells and thymocytes in studies of Gli1/−/− mice (26, 27). Our findings support the idea that different Gli factors function in different contexts (28), and detailed analyses of Gli1 may help further reveal such diverse functions of Gli factors in various developmental processes.

In the past few years, accumulating evidence has led to determination of the transcription factors that mediate the function of Ihh signaling in osteogenesis (29). Runx2, a master regulator of osteogenesis, is thought to play a pivotal role in Hh-mediated
osteogenesis because Runx2 expression is absent in the perichondrium of the Ihh\(^{-/-}\) fetus (7, 20). However, Tu et al. (30) recently reported that recovery of Runx2 expression in skeletal cells does not restore bone formation in Ihh\(^{-/-}\) embryos, suggesting that additional factors besides Runx2 mediate functions of Ihh signaling in the induction of osteoblast differentiation. Indeed, ALP activity and expression of early osteoblast-related genes, including Alp and Bsp, have been observed in the Runx2-positive cells, whereas none of these genes is expressed in the Ihh\(^{-/-}\) perichondrium (7, 20).

Our findings support this notion and further suggest that Runx2 is involved in certain stages of Hh signaling-mediated osteoblast differentiation. Indeed, ALP activity and expression of early osteoblast-related genes, including Alp and Bsp, have been observed in the Runx2-positive cells, whereas none of these genes is expressed in the Ihh\(^{-/-}\) perichondrium (7, 20). Our findings support this notion and further suggest that Runx2 is involved in certain stages of Hh signaling-mediated osteoblast differentiation. Additionally, Hh signaling is known to sequentially induce osteogenesis in both early and late differentiation (20, 31). Our data show that SAG induced both early and late osteoblast marker genes, as well as Runx2. Remarkably, SAG promoted early osteoblast differentiation, at least to some extent, in a Runx2-independent manner; however, a late marker gene was not induced by SAG in Runx2\(^{-/-}\) cells. Overall, Runx2 does not appear to be important in the Hh signaling-mediated specification of the osteoblast lineage, but it is important in subsequent late osteoblast differentiation.

With regard to the contribution of Gli transcription factors in osteogenesis, disruption of Gli3 partially rescued the impairment of early osteoblast differentiation in Ihh\(^{-/-}\) embryos, as shown by the partial recovery of Alp and Runx2, but not Bsp or Oc, in the perichondrium or impaired bone collar formation (13, 14, 32). It has previously been shown that skeletal phenotypes of Ihh\(^{-/-}\) embryos were completely rescued in Ihh\(^{-/-}\);Gli3\(^{-/-}\);C2\(Δ\)NGLi2 embryos, in which C2\(Δ\)NGLi2 (an N-terminally truncated form of GLi2, as a constitutively active form of GLi2) was exogenously expressed in Col2a1-positive cells (14). On the basis of these findings, Joeng and Long (14) suggested that the GLi2 activator and the Gli3 repressor collectively mediate all major aspects of Ihh function in endochondral ossification. Our findings demonstrate how the function of Gli1 is combined with the model of Joeng and Long to allow specification of the osteoblast lineage in the perichondrium.

Gli1 was present in Ihh\(^{-/-}\);Gli3\(^{-/-}\);C2\(Δ\)NGLi2 mice, although it was not detectable in Ihh\(^{-/-}\);Gli3\(^{-/-}\) mice (14). These results

FIGURE 6. Gli1-mediated transactivation of Alp. A, deletion analyses using luciferase reporter constructs containing the 5′-regulatory region of Alp. C3H10T1/2 cells were transfected with a series of deletion fragments in combination with the indicated plasmids. The arrow denotes the major downregulation of luciferase activity by deletion. RLU, relative light units. B, deletion and site-directed mutagenesis analyses for Gli1-responsive elements in Alp. del(-80/-40), deletion mutant of Gli1-responsive elements; m1 and m2, site-directed mutants of Gli1-responsive elements. C, EMSA for the specific binding of GLI1 to GLI1-responsive elements in Alp. Nuclear extracts of C3H10T1/2 cells transfected with GFP or GLI1 were used. The arrowhead indicates shifted bands of the GLI1-DNA complex. D, ChIP assay in the perichondrial cells transfected with the indicated plasmids. DNA was amplified by primer sets spanning (-220 to -21) and not spanning (-1000 to -801) the Gli motif-containing region in the murine Alp (mAlp) region after immunoprecipitation with anti-GLI1 antibody, IgG (negative control), or anti-acetyl histone H3 antibody (positive control), as well as input DNA. For A and B, data are means ± S.D. of triplicate wells, and representative data of independent experiments are shown.
FIGURE 7. Relationship between Gli1, Gli2, Gli3, and Hh input in osteogenesis. A, Gli1 mRNA expression determined by real-time RT-PCR in perichondrial cells from three pairs of WT, Gli2−/−, and Gli3−/− mouse embryos (E17.5). Cells were treated with SAG (1 μM) for 3 days. B, von Kossa staining of the representative sections of femurs, tibias, and metatarsals obtained from two pairs of WT, Gli1−/−, Gli2−/−, and Gli1−/−;Gli2−/− newborn mice. Scale bars = 100 μm. C, in situ hybridization (Col1a1) and immunohistochemistry (Osx) of the representative sections of metatarsals obtained from two pairs of WT, Gli1−/−, Gli2−/−, and Gli1−/−;Gli2−/− newborn mice. Scale bars = 100 μm. D, ALP staining and Alp mRNA expression determined by real-time RT-PCR in perichondrial cells from three pairs of WT, Gli3−/−, and Gli1−/−;Gli3−/− mouse embryos (E17.5). Cells were treated with SAG for 7 days for ALP staining and for 3 days for real-time RT-PCR analysis. *, p < 0.05. For A and C, data are means ± S.D. of triplicate wells, and representative data of independent experiments are shown.
suggested that complete rescue of the Ihh−/− phenotype in Ihh−/−;Gli3−/−;C2ΔNGli2 mice was caused, at least in part, by the recovery of Gli1 in addition to the recovery of Runx2 and the function of the Gli2 activator itself. Furthermore, in this study, we demonstrated that Gli1, as well as Hh input, can promote early osteoblast differentiation, at least to some extent, in a Runx2-independent manner. Taken together, these results suggest that the absence of the Runx2-independent function of Gli1, in addition to the function of Gli2 and Gli3, may account for inadequate rescue of osteoblast phenotypes by the recovery of Runx2 alone in Ihh−/− embryos (30).

In late osteoblast differentiation, Gli2 and Gli3, but not Gli1, are likely to play pivotal roles. Several studies have suggested interactions of Gli2 and Gli3 with Runx2 in late osteoblast differentiation. Shimoyama et al. (33) reported that Gli2 stimulates Runx2 expression, interacts with Runx2, and enhances its activity. The repressor form of Gli3, which was derepressed by Hh input, was previously shown to inhibit Runx2 function by binding to its DNA-binding sites (12). In contrast, no functional interaction between Runx2 and Gli1 was reported so far (12). Our study further shows that expression of Runx2 and Oc was enhanced by SAG, but not by Gli1 overexpression, in WT perichondrial cells, and no expression of Runx2 was observed in the perichondrium of the Gli1−/− fetus. Collectively, these results suggest that Gli1 acts as a permissive factor for the expression of Runx2 at a certain point of development or is indirectly involved in its expression, which also underlies the osteogenic function of Gli1.

In conclusion, this study shows that Gli1 functions collectively with Gli2 and Gli3 in the Hh-mediated specification of the osteoblast lineage. Gli1 plays a redundant role with other Gli activators and is partially involved in the actions of Gli repressors. It remains to be elucidated whether all members of the Gli family function independent of each other at different stages or whether they act collectively at a particular stage in osteogenesis. However, our study provides insight into the elaborate molecular framework of context-dependent differences in Hh-Gli signaling during development, as well as insight into potential clinical applications of Hh-Gli signaling for the treatment of bone defects and non-unions in fracture repair.

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