

# Transcriptional control of Shh/Ptc1 signaling in embryonic development

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## Abstract

In vivo profiling of signal-directed gene expression patterns is a major bottleneck in studying developmental biology. A signal molecule initiates its specific gene expression pattern through the activation of certain transcription factor (TF); however, tissue heterogeneity often masks this pattern due to intercellular complexity of other signal transduction pathways. To decipher the synergistic regulation of signal-directed gene expression in the tissue level, we report here a unique transcriptional responsive element (TRE) existing in the 5'-upstream promoter regions (5'-UPR) of the genes responding to the *Shh/Ptc1* signal transduction pathway during feather placode development in chicken embryos. By locating the TRE homologue and its interactive TF, we were able to reveal the gene expression pattern of the *Shh/Ptc1* signaling. We firstly demonstrated that homology profiling of the 5'-UPR of the genes, *Gli1*, *TGF-β2* and *Msx2*, responding to the *Shh/Ptc1* signaling showed a more than 70% conserved region. Computer alignment of the consensus sequences in the conserved region revealed a 37-nucleotide TRE sequence, containing two regulatory elements homologous to human and mouse Gli-binding sites. Activation of this newly identified Shh/Ptc1-responsive TRE by active Smo signaling in chicken hepatocellular carcinoma cells elicited a strong synergistic expression of the Shh/Ptc1-downstream genes. Based on previous bioinformatics and the present experimental findings, we successfully established an in vivo signaling model for the Shh/Ptc1-directed embryonic feather morphogenesis.

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**Keywords:** Sonic Hedgehog (Shh); Patched-1 (Ptc1); Transcriptional regulatory element (TRE); Gli-binding site; RNA-polymerase cycling reaction (RNA-PCR); Embryonic development

## 1. Introduction

Gene promoters are surrounded by various transcriptional regulatory elements (TRE) in response to the activation of specific transcription factors (TF) mediated through a signal transduction pathway. Upon activation by a signal, the TF directly binds to its responding TRE and triggers the assembly of transcriptional machinery on the specific promoters, initiating the transcription of the signal-responding genes. A signaling pathway is able to stimulate slightly different gene

expression patterns depending upon the presence of various TF and TRE interactions; however, little is known about the synergistic control of the TF-TRE interaction in a specific tissue. To this aim, we hypothesized that a TRE homologue must present in the 5'-upstream promoter regions (5'-UPR) of the signal-responding genes in order to receive the signal stimulation. We have tested this hypothesis by identifying a consensus TRE region in the 5'-UPR of the known Shh/Ptc1-responding *Gli1*, *TGF-β2* and *Msx2* genes expressed during embryonic chicken feather development (Lin, 2002). In this study, we further confirmed its function in response to the Shh/Ptc1 signaling pathway in vitro, demonstrating the synergistic interaction of the TRE to the Shh/Ptc1-specific TF, Gli. After the completion of Human Genome Project in April 2003, the 5'-UPR of numerous genes have been decoded; nevertheless, the identity and location of the relative TRE remain to be determined. Our present study may also facilitate the identification of signal-directed TRE in the human genome.

**Abbreviations:** Shh, Sonic Hedgehog; Ptc, patched; Smo, smoothened; Gli, glioblastoma; TGF-β2, transforming growth factor β2; Tbx, T box family member protein; Msx, muscle segment homologue; Lmx, homeodomain protein; Smad, mothers against DPP homologue; TF, transcription factor; TRE, transcriptional responsive element; 5'-UPR, 5'-upstream promoter region; RNA-PCR, RNA-polymerase cycling reaction.

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Embryonic development requires signal transduction to stimulate tissue-specific gene patterning, which is involved in *in vivo* coordination of cell proliferation, differentiation, migration and programmed cell death (apoptosis) leading to normal organogenesis. *Sonic hedgehog* (*Shh*) is one of the earliest signal genes stimulating embryonic organogenesis, including the formation of limb, eye, wing, central nerve system and skin appendages (Johnson and Scott, 1998; Chuong et al., 2000). In feather, a kind of skin appendages, its expression begins at about the time that feathers start to evaginate from the two-dimensional skin surface, whereas Patched-1 (*Ptc1*), an antagonistic receptor for the *Shh* signal, inhibits this evagination effect (Ingham et al., 2000). *Shh* signaling promotes the proliferation of placode cells in the early feather buds of an E7 (stage 30) chicken embryo, yet is associated with apoptotic recession in the barb marginal plates on the feather filament of an E12 (stage 37) chicken embryo (Ting-Berreth and Chuong, 1996). Regulatory anomalies related to the *Shh/Ptc1* signaling pathway have been reported as a cause for developmental cephalo-defects (Chiang et al., 1996) and cancers, e.g. basal cell carcinoma (Johnson et al., 1996; Oro et al., 1997; Xie et al., 1998) and medulloblastoma (Xie et al., 1997) in human. In *Drosophila*, the binding of *Shh* to *Ptc*, releases smoothed (*Smo*), which is usually inactivated by the *Ptc*, and in turn the free *Smo* phosphorylates *Cos2* and *Fu* and also inhibits the *PKC* to prevent the cleavage of glioblastoma (*Gli*), a transcription factor specific for the *Shh* signaling pathway. The activation of intact *Gli* function stimulates *Shh*-associated downstream gene expression, resulting in cell proliferation, differentiation and apoptosis in various tissues (Kinzler and Vogelstein, 1990; Johnson and Scott, 1998). Since the feather morphogenesis involves very complicated but highly regulated cell proliferation, differentiation and apoptosis within a limited area (a few hundred cells), it is of interest to understand how the *Shh* signaling synergistically and precisely coordinate these downstream phenomena. The mechanism underlying this synergistic regulation of signal-directed gene transcription most certainly will be crucial for the formation of many other organ systems as well.

To decipher the transcriptional control of *Shh/Ptc1* signaling in the formation of embryonic skin appendages, we have developed a molecular screening strategy to detect signal-directed gene expression at the single-tissue scale (Lin, 2002). We applied embryonic chickens with short-term antisense oligonucleotides directed against either *Shh* or *Ptc1*, and then profiled the transcriptional changes of their downstream genes within specific feather placode area in relation to future feather morphogenesis. Single-cell mRNA libraries were amplified ( $\geq 10^7$  folds) from micro-dissected placodes by RNA-polymerase cycling reaction (RNA-PCR) and used for gene-array analysis to detect altered gene expressions in response to the *Shh/Ptc1* knockouts. Seven genes were found and confirmed by Northern blot analysis, fully corresponding to both *Shh* and *Ptc1* signaling, including *Gli1*, *TGF- $\beta$ 2*, *Msx2*, *Tbx4*, *Lmx1*, *Smad3* and *Smad7*. Further, several morphogen genes displayed a partial relationship with either *Shh* or *Ptc1* signaling, indicating a cross-talking network mechanism between members of the *Hh/Ptc* pathways and morphogens of other signaling path-

ways, i.e. *Wnt* and  $\beta$ -*catenin*. Based on this preliminary study, we reported here that homology profiling of currently available *Gli1*, *Tgf- $\beta$ 2* and *Msx2* promoters in the chicken genome showed a conserved TRE homologue of human *Gli*-binding sites, capable of responding to the *Shh/Ptc1* signal transduction pathway during feather placode development in chicken embryos.

## 2. Materials and methods

### 2.1. Oligonucleotides

Synthetic primers used for mRNA amplification were listed as follows: a poly(dT)<sub>24</sub> primer (5'-TTTTTTTTTT TTTTTTTTTT TTTT-3') and an oligo(dG)<sub>6</sub>N-T7 promoter primer (5'-CCAGTGAATT GTAATACGAC TCACTATAGG GAAGGGGGGN-3', N=A, T and C). We applied the poly(dT)<sub>24</sub> primer in the reverse-transcription of poly(A<sup>+</sup>) mRNAs to form the first-strand antisense cDNAs, and then the oligo(dG)<sub>6</sub>N-T7 promoter primer was used as a sense-strand primer for the second-strand cDNA synthesis starting from the complementary poly(dC) tails of the first-strand cDNAs, thus incorporating a T7 RNA promoter in the sense direction of all double-stranded cDNAs. The poly(dC) tails in the 3'-end of the first-strand cDNA were generated by MMLV-like reverse transcriptases. All RNA-PCR primers were RNase-free and purified by polyacrylamide gel electrophoresis (PAGE) before use. For cloning *Smo* carboxy-proximity  $\Delta$ [nts1491–2395], we amplified the sequence directly from the cDNAs of chicken skin explants using PCR primers 5'-dephosphorylated ACTGGCATCT CCATGAGCAC-3' and 5'-dephosphorylated TGCAGGGCTC AGAAGTCTAAG-3'. For cloning *Tgf- $\beta$ 2* genomic 5'-UTR promoter, we sheared chicken genomic DNAs by agitated pipetting and performed PCR using 5'-TCTCCATGGT TTCAGCTGC-3' and 5'-TGCCAGCAGA TAACATCACG-3'.

### 2.2. RNA-polymerase cycling reaction (RNA-PCR)

The following mRNA amplification protocol was based on a published RNA-PCR procedure (Lin et al., 1999; Huang et al., 2003). About 100 microdissected tissue cells (containing 200–500 pg total RNAs) were ruptured by mixture with 20  $\mu$ l of ice-cold Cell Lysis II buffer (Ambion, Austin, TX) and incubation at 75 °C for 10 min. Next the crude cell lysate was treated with DNase I (0.04 U/ $\mu$ l) at 37 °C for 5 min and then 75 °C for 5 min. A quarter of the crude cell lysate was applied to a reverse transcription (RT) reaction (20  $\mu$ l) on ice, comprising 4  $\mu$ l of 5 $\times$  RT buffer (250 mM Tris-HCl, pH 8.3 at 25 °C, 375 mM KCl, 15 mM MgCl<sub>2</sub>, 100 mM DTT), 1  $\mu$ M poly(dT)<sub>24</sub> primers, dNTPs (0.5 mM each for dATP, dGTP, dCTP and dTTP) and RNase inhibitors (40 U). After Superscript II reverse transcriptase (100 U; Invitrogen, Carlsbad, CA) was added, the reaction was incubated at 42 °C for 1 h and shifted to 65 °C for further 10 min. The function of Superscript II reverse transcriptase was not only to synthesize the first strand of cDNA but also to generate a short polymeric dC-tail in the 3'-end of the first-

strand cDNAs for RNA promoter incorporation. The first-strand cDNA–RNA hybrids so obtained were then denatured at 94 °C for 3 min and instantly mixed with 1  $\mu$ M oligo(dG)<sub>6</sub>N-T7 promoter primer at 37 °C for 5 min. Half (10  $\mu$ l) of the denatured first-strand cDNAs was added into a double-stranding (DS) reaction (40  $\mu$ l) on ice, containing 4  $\mu$ l of 10 $\times$  DS buffer (500 mM Tris–HCl, pH 9.2 at 25 °C, 160 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 27.5 mM MgCl<sub>2</sub>), dNTPs (0.5 mM each for dATP, dCTP and dTTP) and *Taq/Pwo* DNA polymerase mixture (total 5 U). The reaction formed promoter-linked double-stranded cDNA amplicon at 37 °C for 5 min and then 68 °C for 25 min. An in vitro transcription (IVT) reaction (40  $\mu$ l) was then set up for mRNA amplification, containing 15  $\mu$ l of the promoter-linked double-stranded cDNA amplicon, 4  $\mu$ l of 10 $\times$  IVT buffer (400 mM Tris–HCl, pH 8.0 at 25 °C, 60 mM MgCl<sub>2</sub>, 100 mM DTT, 20 mM spermidine), rNTPs (2 mM each for ATP, GTP, CTP and UTP) and T7 RNA polymerase (200 U; Epicentre, Madison, WI). After 2-h incubation at 37 °C, the resulting RNA transcripts were purified by RNA affinity resin (i.e. RNeasy spin columns, Qiagen) and directly used for another round of amplification through cycling steps of the above RT, DS and IVT. The quality of second-amplified RNA library (2  $\mu$ g) was assessed on a 1% formaldehyde-agarose gel. Labeling of the resulting cDNAs was achieved by the incorporation of labeled nucleotides or analogs during RT, while that of resulting RNAs was accomplished during IVT.

### 2.3. TRE identification by promoter alignment

The 5'-upstream promoter regions (5'-UPR) of avian *Gli1*, *TGF- $\beta$ 2* and *Msx2* genes were collected from the NCBI online resources (<http://www.ncbi.nlm.nih.gov>), the Ensembl database (<http://www.ensembl.org>) and the UCSC genome bioinformatics (<http://genome.ucsc.edu>). We screened homologous regulatory elements in these 5'-UPR sequences using a sequence alignment utility program provided by the Baylor College of Medicine HGSC (<http://dot.imgen.bcm.tmc.edu:9331/seq-search/alignment.html>). This software was an enhanced version of the NCBI's BLAST nucleotide search tool that facilitated identification and clustering of the conserved regions among multiple sequence alignments (Worley et al., 1998). The software also offered a pre-set analysis for the locations and functions of the conserved regions within the Entrez database, which was convenient for the new and infrequent users. Based on this simple but useful bioinformatic analysis, we selected the conserved regions sharing more than 70% sequence homology as potential candidates for chicken *Shh*/*Ptc1*-responsive TRE. After we experimentally tested each TRE homologue in response to the *Shh*/*Ptc1* signaling in vitro, the most effective TRE was termed to be *Shh-RE*.

### 2.4. Vector construction for testing TRE functionality

Two oligonucleotide inserts, *Smo*- $\Delta$ [nts1491–2395] (AF01997) and *TGF- $\beta$ 2*-promoter- $\Delta$ [nts194–925] (X58071), were prepared by PCR and confirmed by oligonucleotide sequencing. First, the *Smo*- $\Delta$ [nts1491–2395] encoding an active

form of amino-terminus-truncated *Smo* protein ( $\Delta$ *Smo*) was ligated to the 3'-*Xba*I restricted site of a mutated red fluorescent *HcRed1* gene, *rGFP*, and then cloned into the *Cla*I site of an avian retroviral vector, RCAS–TFA (Widelitz et al., 2000). This formed a replication-incompetent RCAS– $\Delta$ SMO vector for constitutively expressing the  $\Delta$ *Smo*–*rGFP* gene in transfected chicken cells. Second, we inserted the chicken *TGF- $\beta$ 2*-promoter- $\Delta$ [nts194–925] into the *Hind*III site of an eGFP-expressing pCMS-EGFP vector (BD Clontech). The *TGF- $\beta$ 2* promoter was used to replace the original CMV promoter and thus to drive the expression of green fluorescent eGFP protein in chicken cells. To test potential Gli-binding sites, the *TGF- $\beta$ 2* promoter was further cleaved by *Afl*I and *Mse*I and then either blunt-ligated to remove the original Gli-responsive element ( $\Delta$ Gli Pr-eGFP), or ligated to a synthetic insert sequence of the newly identified *Shh-RE* (Gli-eGFP) as shown in Fig. 1B. Both vectors were propagated in *Escherichia coli* DH5 $\alpha$  LB-culture containing either 100  $\mu$ g/ml neomycin (for RCAS– $\Delta$ SMO) or 50  $\mu$ g/ml ampicillin (for Gli Pr-eGFP). Vectors were transfected (20 nM) into tested chicken hepatocellular carcinoma LMH cells using Fugene reagent (Roche Biochemicals) according to manufacturer's recommendations. For virus packaging, the RCAS– $\Delta$ SMO plasmid was transfected into chicken embryonic fibroblast (CEF) cells grown in 100-mm plates at 37 °C in a humidified, 5% CO<sub>2</sub> atmosphere in Waymouth's MB752/1 containing 10% FBS, using Fugene reagent. Virus-containing medium was harvested 24 h post-transfection. Filtered-virus-containing medium with 4  $\mu$ g/ml hexadimethrine bromide (Sigma) was used to infect chicken LMH cells at a multiplicity of infection (MOI) of 5 viral particles per cell. Infected cells will be grown for 48 h before subsequent experiments. Vector aliquots were stored at –80 °C until needed for the next transfection.

### 2.5. Cell culture and transfection

Chicken hepatocellular carcinoma LMH cells acquired from ATCC were grown in Waymouth's MB752/1 medium supplemented with 10% fetal calf serum at 37 °C with 5% CO<sub>2</sub>. The cells were grown on 0.1% gelatin-coated dishes and 75% of the medium was replaced with new growth medium every 48 h. Cultures were passaged at ~80% confluency by exposing cells to 1 $\times$  trypsin–EDTA solution (Irvine Scientific) for 1 min and detached cells were replated at 1:10 dilution in fresh growth medium. At 24 h prior to the first transfection, the fetal bovine serum was reduced to 2%. The cells at 40–50% confluency were first transfected with RCAS– $\Delta$ SMO as aforementioned. About 12 h later, the Gli1 Pr-eGFP plasmid (50  $\mu$ g) was dissolved in 50  $\mu$ l DOTAP liposomal reagent (Roche) and used to transfect the cells. Cellular protein samples were isolated 2 days after the second transfection and tested for Western blotting and ELISA analyses.

### 2.6. Western blotting and ELISA analyses

Cells were rinsed with ice-cold PBS twice and then treated with the CelLytic-M lysis/extraction reagent (Sigma Chemical,



transferred onto nylon membranes (Schleicher and Schuell, Keene, NH). Probes was random hexamer-labeled with the Prime-It II kit (Stratagene, La Jolla, CA) in the presence of [<sup>32</sup>P]dATP (>3000 Ci/mM, Amersham International, Arlington Heights, IL), and purified with 30 bp-cutoff Micro Bio-Spin chromatography columns (Bio-Rad). Hybridization was carried out in a mixture of 50% freshly deionized formamide (pH 7.0), 5× Denhardt's solution, 0.5% SDS, 4× SSPE and 250 μg/μL denatured salmon sperm DNAs (18 h, 42 °C). Membranes were sequentially washed twice in 2× SSC, 0.1% SDS (15 min, 25 °C), and once in 0.1× SSC, 0.1% SDS (15 min, 65 °C) before autoradiography.

### 2.8. Statistical analysis

Results were presented as mean±S.E.M. Statistical data comparison was performed by one-way ANOVA. When significant differences were found, the Dunnett's method was used to identify the data sets that differed from the control data. Probability values of  $p < 0.05$  were considered significant. All  $p$  values were determined from two-sided tests.

## 3. Results

### 3.1. Identification of homologous Gli-binding sites in Shh/Ptc1-responsive promoters

Synergistic regulation of signal-directed gene expression is very crucial for normal organogenesis. Signal molecules involved in cell proliferation, differentiation, migration and apoptosis are stimulated at precise time and location to sculpt organ morphology and therefore develop correct biological function. Dysregulation of these signal genes has been frequently observed to cause organ deformity and dysfunction during embryonic development and cancers in adult. For instance, regulatory anomalies related to the *Shh/Ptc1* signal transduction pathway have been found to cause developmental cephalo-defects and cancers, including basal cell carcinoma and medulloblastoma in human (Hahn et al., 1996; Dahmane et al., 1997). To understand the normal expression pattern of the Shh/Ptc1 signaling pathway, we have identified Shh/Ptc1-downstream genes using microdissection, RNA-PCR and gene-array analyses (Lin, 2002). In embryonic development of chicken feathers, seven primary downstream genes were confirmed to fully respond to both Shh and Ptc1 signals, including *Gli1*, *TGF-β2*, *Msx2*, *Tbx4*, *Lmx1*, *Smad3* and *Smad7*. For synergistic regulation of these Shh/Ptc1-responding genes, a common regulatory element in the 5'-upstream promoter regions (5'-UPR) of these genes was proposed to be required. Since the *Shh/Ptc1* signal transduction pathway has known to function through the activation of Gli transcription factors, the Gli-binding sites likely serve as one of the regulatory elements in the 5'-UPR.

The *Gli* family members are zinc finger-containing proteins encoded by homologues to the *Drosophila* segment polarity gene cubitus interruptus. The Gli transcription factors share highly conserved homology in their gene sequences and tend to interact with G/C-rich motifs in an RNA promoter through

their zinc fingers (Kinzler et al., 1988; Marigo et al., 1996). Although it is well known that human Gli-binding sites are highly conserved, sized about 9–11 bp and of high G–C content (Liu et al., 1998; Dai et al., 1999), the identification of chicken counterparts remains to be determined. We therefore collected the 5'-upstream promoter regions (5'-UPR) of currently known avian *Gli1*, *TGF-β2* and *Msx2* genes from the NCBI online resources, the Ensembl database and the UCSC genome bioinformatics, and then analyzed them using a sequence alignment utility program provided online by the Baylor College of Medicine HGSC, which not only facilitated identification and clustering of the conserved regions among multiple sequence alignments (Worley et al., 1998) but also dramatically reduced the time and cost involved. Because conserved regions were bound by proteins more frequently than non-conserved regions (Bulyk, 2003; Siggia, 2005), we selected the conserved regions sharing more than 70% sequence homology as potential candidates for chicken Shh/Ptc1-responsive TRE.

By computer comparing sequence alignments among the 5'-UPR of the known Shh/Ptc1-responding genes, we found a series of 37-nucleotide (nts) consensus TRE sequences with an average of 74% homology (Fig. 1A). These consensus TRE were located about 161 nts up-stream to the transcription initiation site of *Gli1*, 294 nts up-stream to that of *TGF-β2* and right before that of *Msx2*. TRE are usually short homologue (5–15 bp) and frequently degenerate sequence motifs that confer different levels of affinity to TF, thus causing some genes to be transcribed at higher levels than others (Bulyk, 2003; Siggia, 2005). To identify the most effective TRE, we experimentally tested each identified TRE and their homologues in response to the Shh/Ptc1 signaling in vitro. The most effective Shh/Ptc1-responding TRE was therefore constructed, showing a conserved oligonucleotide sequence of 5'-GCTACCGGAGGGCCGCGTCC TGCCTGGTCT GCACGTA-3' (*Shh-RE*) (Fig. 1B). Interestingly, this TRE homologue contains two G/C-rich motifs which were highly homologous to the published human and mouse Gli-binding sites (Dai et al., 1999; Liu et al., 1998), including homologues of 5'-CGCGTCCTG-3' and 5'-TGCCTGGTC-3'. Given that Gli, a conserved transcription factor family among different animal species, is known to stimulate Shh/Ptc-associated downstream gene expression, resulting in cell proliferation, differentiation and apoptosis in various tissues during development (Kinzler and Vogelstein, 1990; Johnson and Scott, 1998), our finding of this signal-responding TRE is very useful for understanding the locations and functions of Gli-TRE interactions in Shh/Ptc1-directed developmental events.

### 3.2. Shh/Ptc1-directed transcription through the identified Shh-RE

Mis-expression of Shh/Ptc signaling has been reported to occur in diverse epithelial cancers, e.g. basal cell carcinoma in mouse and human (Hahn et al., 1996; Dahmane et al., 1997). Activation of Shh signaling by mutated Smo, an agonist of Shh signal molecules, is one of carcinogenic mechanisms. By co-

transfecting a truncated form of active Smo ( $\Delta$ Smo) and a *Shh-RE*-containing reporter vector (pCMS-EGFP) into chicken carcinoma LMH cells (Fig. 2A), the  $\Delta$ Smo was found to stimulate Shh/Ptc1-downstream genes, including the green fluorescent reporter gene, *eGFP*. The *eGFP* expression was driven by a recombinant *Shh-RE*-containing *TGF- $\beta$ 2* promoter (Gli Pr), in which the original Gli-binding site was replaced by *Shh-RE*, indicating that this Shh/Ptc1-responsive TRE is able to receive the Shh signal and to activate the relative transcriptional control.

On the other hand, the success of  $\Delta$ Smo activation was demonstrated by co-expression of a red fluorescent protein, rGFP, which was designed to replace the transmembrane domains of wild-type Smo and thus to release the inhibitory effect of Ptc1 on the  $\Delta$ Smo-rGFP fusion protein. Neither Gli Pr-driven *eGFP* itself without  $\Delta$ Smo nor defective Gli Pr ( $\Delta$ Gli Pr) with  $\Delta$ Smo can stimulate the expression of the *eGFP* reporter gene, suggesting that *Shh-RE* is required for the tran-

scriptional control of the Shh/Ptc1 signal transduction pathway. Both Western blot (Fig. 2B) and ELISA (Fig. 2C) analyses of  $\Delta$ Smo-rGFP vs. Gli Pr-driven *eGFP* also confirmed the results of Fig. 2A. Thus, this newly identified TRE contains at least a common regulatory element in response to the Shh/Ptc1 signaling pathway. The finding of avian Gli-binding sites in this Shh/Ptc1-responding TRE sequence has further proven the Gli-*Shh-RE* interaction, which may facilitate the *in vivo* research of Shh/Ptc1-directed gene expression patterning during embryonic development in chicken.

### 3.3. Synergistic expression of *Shh/Ptc1*-downstream genes in response to the Gli Pr activation

Following the transcriptional activation of the *Shh-RE* by  $\Delta$ Smo in chicken carcinoma cells, we observed the synergistic expression of Shh/Ptc1-downstream genes, similar to the in

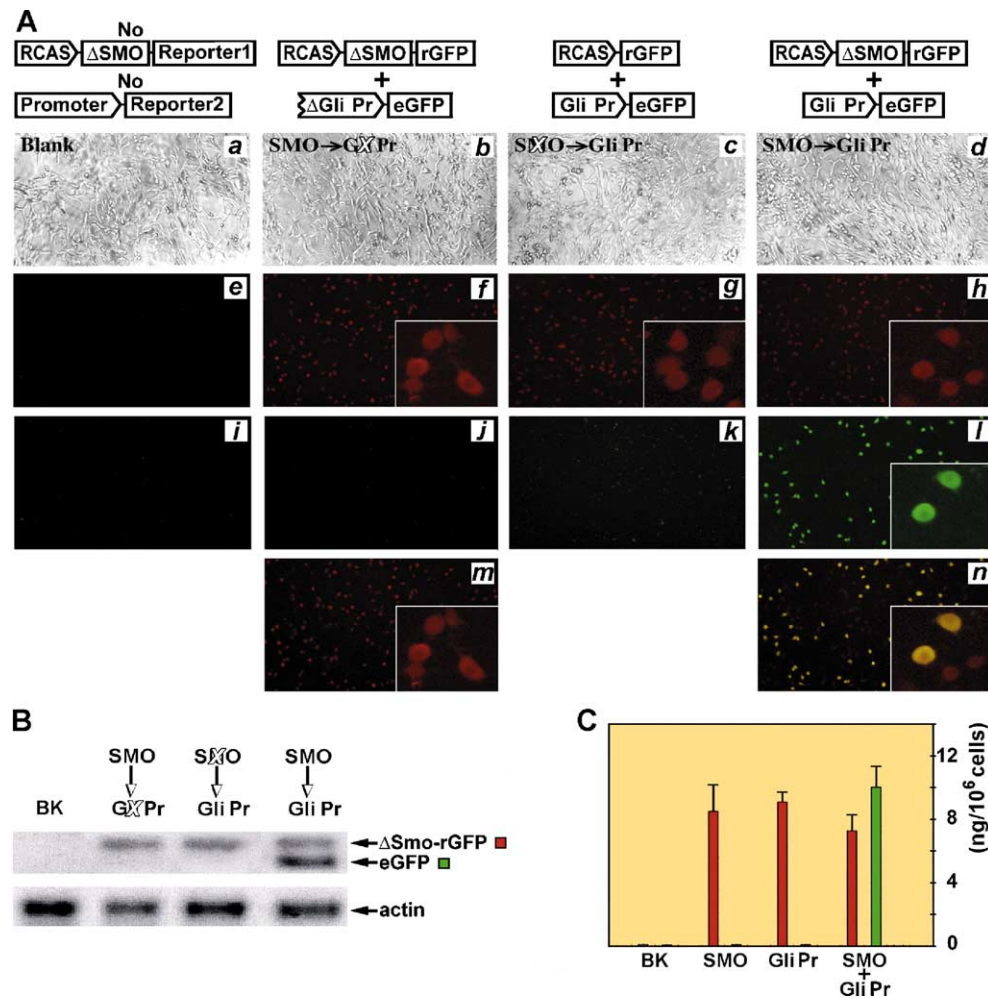


Fig. 2. Transcriptional activation of the identified Shh/Ptc1-responsive TRE (*Shh-RE*) in chicken hepatocellular carcinoma cells, LMH. (A) After activation of Shh signaling by transfecting the RCAS- $\Delta$ SMO vector ( $\Delta$ Smo) into the carcinoma cells, the *Shh-RE*-containing promoter (Gli Pr) was able to trigger the transcription of its downstream reporter gene, green fluorescent *eGFP* (l, n). Transfection of the active  $\Delta$ Smo was demonstrated by co-expression of a red fluorescent protein, rGFP, which was designed to replace the transmembrane domains of wild-type Smo and thus to prevent the inhibitory effect of Ptc1 on the  $\Delta$ Smo-rGFP fusion protein (f, h, m, n). Neither Gli Pr-driven *eGFP* itself without  $\Delta$ Smo activation (g, k) nor defective Gli Pr ( $\Delta$ Gli Pr) with active  $\Delta$ Smo (f, j, m) can stimulate the expression of *eGFP*, suggesting that the Gli Pr is required for the transcriptional control of the Shh/Ptc1 signal transduction pathway. No detectable change in cell morphology was observed (a–d). Both Western blot (B) and ELISA (C) analyses of  $\Delta$ Smo-rGFP vs. Gli Pr-driven *eGFP* expressions confirmed the results of (A), showing that only a promoter containing the *Shh-RE* can respond to the *Shh* signaling pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vivo expression pattern in the early embryonic feather development, including *Gli1*, *TGF- $\beta$ 2*, *Msx2*, *Tbx4*, *Lmx1* and *Smad3*, but not *Smad7* (Fig. 3). This suggests that a signal transduction pathway is able to trigger the same TF–TRE interaction and to activate similar downstream gene expression in various types of cells and tissues. This finding also indicates that both developmental embryos and cancer cells can adopt the same signaling mechanism to synergistically regulate the signal-downstream gene stimulation; however, the timing and localization of signal transduction may determine the results of cell proliferation, differential, migration and apoptosis. Given that TRE are usually short degenerate sequence motifs conferring different levels of affinity to certain specific TF (Bulyk, 2003; Siggia, 2005), the changes of single nucleotide polymorphism (SNP) in the TRE region may explain the slightly different gene expression patterns between a developmental organ and a cancerous tissue. However, such a slight difference in the signal-directed gene regulation is sufficient to cause the deformity and dysfunction of normal organ development.

In the previous study of embryonic chicken development (Lin, 2002), we have observed that several morphogen genes displayed only partial relationships with either Shh or Ptc1 signaling, i.e. *Wnt*,  $\beta$ -catenin and BMP. These pathways may also play an important role in adjusting the timing and localization of the Shh/Ptc1 signaling for precisely embryonic development. Thus, it is understandable that cancer cells will more likely give rise from normal tissues when the other regulatory signaling mechanisms are lost or dysregulated. To this aim, because the resolution of individual signaling pathway is still under investigation, the cross-talking interactions among dif-

ferent pathways are currently beyond our reach. A gene promoter may contain various TRE for demonstrating its capability in receiving different signals. Our present approach has therefore paved an easy way to search different signal-specific TRE in the proximity of a gene promoter, which in turn provide a clue for understanding the effects of signal–signal interactions on the gene.

Current organogenesis research focuses on the transcriptional control of signal-directed gene expression. Following the synergistic regulation of natural gene patterning, we may be able to modulate the progress of developmental events and thus to maintain the organ normality. The synergistic expression of Shh/Ptc1-downstream genes was regulated by the activation of intact Gli3 and further maintained by Gli1 in chicken feather placodes (Lin, 2002). Gli1 has been reported as a positive regulator for maintaining Shh signaling in neural tube development (Lee et al., 1997), while Gli3 is a composite of positive (C-terminal) and negative (N-terminal) regulatory domains to the Shh signaling (Sasaki et al., 1999). Without activation by the Shh signaling, the Gli3 is cleaved and its N-terminal prevents the access of Gli-binding sites (Aza-Blanc et al., 1997; Buscher et al., 1997). Since the expression of Gli3 rather than Gli2 was highly abundant during Shh signaling and Gli1 has been proven to be one of the Shh/Ptc1-directed downstream genes (Fig. 3A), the intact Gli3 may play the role of active TF in binding to the Shh/Ptc1-responsive TRE for downstream gene stimulation in embryonic feather formation. This finding has also been observed in other development systems (Marigo et al., 1996; Dai et al., 1999). However, how Shh signaling preserves the intact Gli3 structure to mask its repressive function remains to be elucidated.

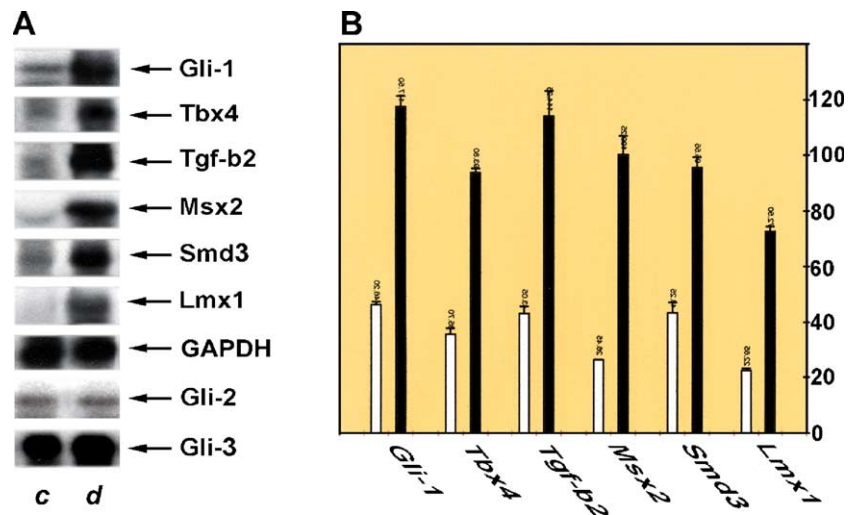


Fig. 3. Co-activation of Shh/Ptc1-downstream genes by  $\Delta$ Smo in response to the Shh/Ptc1-responsive TRE. (A) Comparison of Northern blotting results from untreated (c) and  $\Delta$ Smo-treated (d) LMH cells in Fig. 2A showed that the Shh/Ptc1-downstream genes were highly stimulated by the active Smo (Shh) signaling, concurrently to the activation of Gli Pr. Because Gli1 was downstream to the Shh/Ptc1 signaling and the expression of Gli3 rather than Gli2 was highly abundant in the feather bud area, these findings indicate that Gli3 may be the transcription factor activated by the Shh/Ptc1 signaling and then interacting with the Gli-binding site for triggering Shh/Ptc1-downstream transcription. (B) Bar chart analysis demonstrated the changes of Shh/Ptc1-downstream genes of (A), including *Gli1*, *Tbx4*, *TGF- $\beta$ 2*, *Msx2*, *Smad3* and *Lmx1*. The relative level of GAPDH expression was set to be 100 of the scale. Since the known 5'-UPR of these Shh/Ptc1-downstream genes contained the identified *Shh-RE* homologues and the removal of this estimated Gli-binding site silenced the Shh-directed gene transcription as shown in Fig. 2, it suggests that Gli3 and probably Gli1 are responsible for synergistic regulation of the Shh/Ptc1-downstream gene expression in embryonic feather development.

### 3.4. Model of the *Shh/Ptc1* signaling pathway in embryonic feather development

Since functions of the *Shh/Ptc1*-downstream genes have been well established in the previous studies, the interaction of these genes in feather morphogenesis can be predicted as an accumulative effect of all gene functions involved within an overlapping frame of time and space. When the current findings and previous observations using in situ hybridization and immunostaining (Ting-Berreth and Chuong, 1996; Nieto et al., 1996) were added, a detailed picture emerged as shown in Fig. 4. This new modeling strategy was based on the time-course and spatial expression patterns of the *Shh/Ptc1*-downstream genes. Our previous studies have shown that the primary *Shh/Ptc1*-downstream genes should be able to respond to not only *Shh* but also *Ptc1* signaling due to their mutually antagonistic functions. Genes responding only to either *Shh* or *Ptc1* were not primary candidates to the *Shh/Ptc1* signaling pathway. According to these criteria, seven genes were identified to be the primary *Shh/Ptc1*-downstream genes, including *Gli1*, *TGF- $\beta$ 2*, *Msx2*, *Tbx4*, *Lmx1*, *Smad3* and *Smad7*. In this study, we suggest that these genes were synergistically regulated by *Gli3* and its binding site, *Shh-RE*. Therefore, the *Shh/Ptc1*, *Gli3* and their downstream genes, *Gli1*, *Tgf- $\beta$ 2*, *Msx2*, *Tbx4*, *Lmx1*, *Smad3* and *Smad7*, together form the backbone of the *Shh/Ptc1* signal transduction pathway.

At the placode stage (*E6*–*6.5*), a positive feedback interaction between early *Shh* and  $\beta$ -catenin signaling was found. As reported by Ting-Berreth and Chuong (1996), the  $\beta$ -catenin signaling seems to precede that of *Shh* to set up a condensation zone for feather bud evagination and, within the zone, *Shh* promotes cell proliferation to induce primitive feather growth out of the skin surface. Without the support of  $\beta$ -catenin signaling, the spatial orientation of feather morphogenesis will be upside-down like hair formation (Lin, 2002). At the short feather bud stage (*E7*–*8* embryo), *Shh* signaling becomes highly activated. Although three members of the *Gli* family, *Gli1*, *Gli2* and *Gli3*, have been well known to be the transcriptional activators of the *Shh/Ptc1* signaling pathway during the organogenesis of various systems (Kinzler et al., 1988; Altaba et al., 2002), we currently showed the roles of *Gli1* and *Gli3*, but not *Gli2*, in the embryonic chicken feather morphogenesis. Because *Gli1* has been confirmed directly downstream to the *Shh/Ptc1* signaling and *Gli3* is abundantly expressed regardless to the *Shh/Ptc1* signaling at this stage, it is most likely that *Gli3* plays a very important role in the transcriptional regulation of the *Shh/Ptc1*-downstream genes. The interaction of *Gli3* and *Shh/Ptc1* TRE triggers the onset of the primary downstream gene expression, which results in fine-tuning further morphogenetic processes. For example, the function of *Gli1* has been reported to prolong the *Shh* signaling effects without the induction of *Shh* (Lee et al., 1997). The *Gli1*-induced continuation of *Shh*-independent cell proliferation may provide the long-term growing force for establishing proximal–distal polarity.

When feather buds continue to grow out of the skin surface, several morphogens of other signaling pathways begin to set up anterior–posterior and proximal–distal polar-

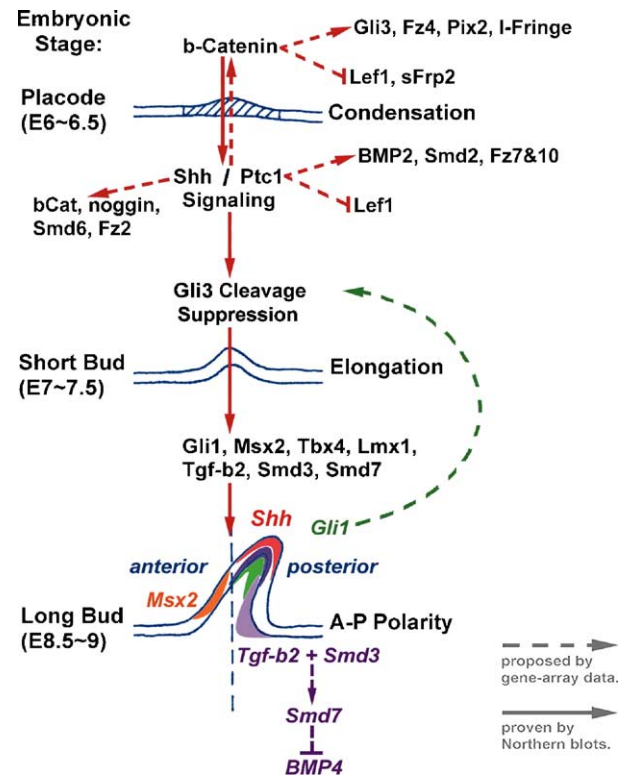


Fig. 4. Functional modeling of the *Shh/Ptc1* signal transduction pathway during embryonic feather morphogenesis. A solid red arrow line indicates that the interactions have been confirmed by both gene-array and Northern blot analyses in our previous studies, while a dotted red arrow line represents a potential pathway detected only by the gene-array analysis. During feather bud condensation ( $\sim E6$ ),  $\beta$ -catenin (bCat) sets up the earliest margin zone for *Shh* signaling. The activation of *Shh/Ptc1*-downstream genes, such as *Gli1*, *Tbx4*, *TGF- $\beta$ 2*, *Msx2*, *Smad3* and *Lmx1*, leads to the formation of early symmetric feather buds ( $\sim E7$ ). The expression of *Gli1* has been shown to prevent the cleavage of *Gli3* and thus extend the *Shh/Ptc1* signaling effect on feather growth. A dotted green arrow line shows the maintenance of long-term *Shh/Ptc1* signaling by *Gli1* stimulation. At the anterior–posterior (A–P) polarity stage of asymmetric long feather bud formation (*E8*–*9*), the distributions of *Shh* (red), *Gli1* (green), *TGF- $\beta$ 2* (dark purple), *Smad3* (pale purple) and *Msx2* (orange) gene expressions are outlined, according to their in vivo localization of immunostaining and in situ hybridization as previously reported. Then, interactions among these *Shh/Ptc1*-downstream genes trigger further downstream signaling networks. For example, the interaction between *TGF- $\beta$ 2* and *Smad3* has been shown not only to activate *Smad7* but also to inhibit *BMP4* expression (purple lines). Moreover, the antagonistic interaction between *Smad7* and *BMP4* has been observed in the previous gene-array analysis, corresponding to both *Shh* and *Ptc1* knockdown effects (Lin, 2002). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ities for formation of future feather shapes. Some of the identified primary downstream genes were found to be involved in this direction. The bottom half of Fig. 4 outlines the distribution of *Shh* (red), *Gli1* (green), *TGF- $\beta$ 2* (dark purple), *Smad3* (pale purple), and *Msx2* (orange) in an *E8*–*8.5* chicken feather bud. Basically, expressions of *Shh*, *Gli1*, *TGF- $\beta$ 2* and *Smad3* are in the posterior region, while that of *Msx2* is in the anterior epithelium. The distribution of *Shh* and *TGF- $\beta$ 2* secretory proteins could extend to a larger area than what is shown here. It is clear that the overlapping zone of *Shh*, *Gli1*, *Tgf- $\beta$ 2* and *Smad3* expressions orients the

direction of feather bud growth. In the meantime, the interaction of TGF- $\beta$ 2 and Smad3 may trigger Smad7 expression (von Gersdorff et al., 2000; Stopa et al., 2000; Brodin et al., 2000; Denissova et al., 2000), an inhibitor of BMP signaling, and thus inhibits BMP signaling in the posterior *E8* feather bud. Because BMP4 is an inhibitor for feather growth (Jung et al., 1998) and our previous data also showed that it functions in opposite to the Shh/Ptc1 signaling (Lin, 2002), the Smad7-induced inhibition of BMP4 may contribute to the fast growth of the posterior feather bud, resulting in establishment of the anterior–posterior polarity. This extends Shh function for asymmetric feather elongation.

#### 4. Discussion

The strategy employed here successfully determines the transcriptional regulation of a signaling pathway in a specific developmental event. Our previous gene-array study has identified seven primary downstream genes to the *Shh/Ptc1* signal transduction pathway (Lin, 2002). In conjunction with peers' studies using time-course *in situ* hybridization and immunostaining (Ting-Berret and Chuong, 1996; Nieto et al., 1996; Morgan et al., 1998), the present finding of Shh/Ptc1-directed TF–TRE interactions has revealed the molecular basis of chicken feather organogenesis, which may be useful for *in vivo* research of other skin appendage formation. By bridging the gap between morphological alterations and molecular signal interactions, we provided an easy way to understand how nature controls the transcriptional processes underlying organogenesis. In this study, we first identified the signal-responsive regulatory elements in the 5'-upstream promoter regions (5'-UPR) of known signal-downstream genes. The 5'-UPR should contain at least one responsive regulatory element specific to the signal in order to receive signal-dependent transcriptional activation. Based on the unique regulatory elements of the identified TRE, we were able to locate potential TF molecules interacting with the TRE.

Although this approach overlooks the fact that some genes may also contain the TRE but not respond to the signal due to other inhibitory effects, such as DNA methylation and histone modification, our findings have shed light on the understanding of TF–TRE interactions in response to a signaling pathway during specific organogenesis. Further research can be developed based on such information. For example, we may analyze the TRE distribution in a genome to identify all potential signal-responding genes and then compare them to a tissue-specific gene expression pattern to profile the gene silencing pattern of DNA methylation and histone modification in the tissue. The complete knowledge of signal-specific TF–TRE interaction, DNA methylation and histone modification will help not only to understand the mechanisms of natural gene regulation in various organogenesis and but also to provide a more reliable therapeutical design for embryonic dysformation diseases. Therefore, learning how to exploit such information in the future research and therapy development will be a forthcoming challenge.

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