Induction of Sonic hedgehog mediators by TGF-β: Smad3-dependent activation of Gli2 and Gli1 expression in vitro and in vivo

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Abbreviations: TGF-β: Transforming growth factor-β; Hh: Hedgehog; Ptc: Patched; Smo: Smoothened
Abstract

Hedgehog (Hh) and TGF-β family members are involved in numerous overlapping processes during embryonic development, hair cycle and cancer. Herein, we demonstrate that TGF-β induces the expression of the Hh signaling molecules Gli1 and Gli2 in various human cell types, including normal fibroblasts and keratinocytes, as well as various cancer cell lines. Gli2 induction by TGF-β is rapid, independent from Hh receptor signaling, and requires a functional Smad pathway. Gli1 expression is subsequently activated in a Gli2-dependent manner. In transgenic mice overexpressing TGF-β1 in the skin, Gli1 and Gli2 expression is also elevated, and depends on Smad3. In pancreatic adenocarcinoma cell lines resistant to Hh inhibition, pharmacologic blockade of TGF-β signaling leads to repression of cell proliferation accompanied with a reduction in Gli2 expression. We thus identify TGF-β as a potent transcriptional inducer of Gli transcription factors. Targeting the cooperation of Hh and TGF-β signaling may provide new therapeutic opportunities for cancer treatment.
Introduction

The Hedgehog (Hh) signaling pathway is critical for stem cell maintenance, embryonic patterning and growth in both invertebrates and vertebrates (1). Deregulation of the Hh pathway is a characteristic trait of a number of pathological states, including developmental syndromes with high proneness to cancer (1, 2). Cellular responses to the Hh signal are controlled by two transmembrane proteins, the tumor suppressor Patched-1 (Ptch) and the oncoprotein Smoothened (Smo) (2, 3). The latter has homology to G-protein-coupled receptors and transduces the Hh signal. In the absence of Hh, Ptch maintains Smo in an inactive state, thus silencing intracellular signaling. With the binding of Hh, Ptch inhibition of Smo is released and the signal is transduced. The transcriptional response to Hh signaling is mediated by a family of zinc-finger transcription factors which comprises the Ci protein in Drosophila, and three closely related Gli proteins in vertebrates: Gli1, Gli2 and Gli3 (4). Gli2 is thought to function upstream of Gli1 and to be the primary mediator of Hh signaling (5), inducing Gli1 expression via direct binding to its promoter region (6). Gli transcription factors regulate multiple cellular functions associated with malignant transformation, such as cell cycle progression and apoptosis (4, 7).

The importance of the Hh signaling pathway in tumorigenesis was established through the discovery of inactivating mutations in the Ptch gene in patients with familial (Gorlin’s syndrome) basal cell carcinomas (BCC) and sporadic BCC (8, 9). Other tumors exhibit inappropriate Hh pathway activation. For example, some esophageal squamous cell sarcomas and transitional cell carcinomas of the bladder may carry loss-of-function mutations of the Ptch gene (10), while gain-of-function mutations of Smo have been identified in a subset of small cell lung carcinoma (11). Additionally, overexpression of the main Hh member Sonic
hedgehog (Shh), leading to activation of Smo, has been identified in some gastro-intestinal cancers (12) and pancreatic adenocarcinomas (13).

Similar to Hh members, Transforming Growth Factor-β (TGF-β) has emerged as a family of growth factors involved in various essential physiological processes that include embryonic development, tissue repair, cell growth control and differentiation. TGF-β isoforms are expressed in a variety of tumor types, and contribute to the aggressiveness and progression of neoplasms (14, 15). TGF-β members signal via membrane-bound heteromeric serine-threonine kinase receptor complexes. In most cell types, TGF-β binds to TβRII in combination with TβRI, also known as ALK-5 (16). Receptor activation by TGF-β leads to phosphorylation of cytoplasmic proteins of the Smad family. Receptor-associated Smads, Smad2 and Smad3 then heteromerize with Smad4, translocate into the nucleus, and act as transcription factors to regulate target gene expression. Smad3 is thought to contribute most Smad-dependent responses to TGF-β in the adult, while Smad2 is critical during embryogenesis (17, 18).

In this study, we have examined the capacity of TGF-β to modulate the expression of the Hh signaling molecules Gli1 and Gli2. We provide definitive evidence, both in vitro and in vivo, for Smad-dependent activation of Gli2 expression, and consequently, that of Gli1, thereby identifying TGF-β as a cytokine ubiquitously capable of activating, enhancing, or prolonging Hh signals. Also, we demonstrate that some cyclopamine-resistant pancreatic adenocarcinoma cell lines are growth inhibited by a small molecule inhibitor of TGF-β signaling.
Materials and Methods

Cell cultures and reagents. Primary human dermal fibroblasts, WI-26 human transformed lung fibroblasts, HaCaT immortalized human keratinocytes, MDA-MB-231, and MDA-MB-468 breast carcinoma cell lines, and PANC-1 human pancreatic adenocarcinoma cells were all maintained in Dulbecco's modified Eagle medium with 10% FBS and antibiotics (Invitrogen, Carlsbad, CA). When indicated, cells were serum-starved for 16 hours and treated with human recombinant TGF-β1 (5 ng/ml, referred to as TGF-β) and/or human recombinant N-terminus Shh peptide (1.5 µg/ml), both purchased from R&D Systems (Minneapolis, MN). Cyclopamine, cycloheximide, and the kinase inhibitors, SB431542, SB203580, PD98059, SP600125, were obtained from Euromedex (Strasbourg, France). LY294002 was from Calbiochem-Merck (Nottingham, UK). siRNAs were purchased from Ambion/Applied Biosystems (Courtabœuf, France) and transfected into cells using the RNAiFect reagent (Qiagen, Courtabœuf, France). Transfection of MDA-MB-468 cells with Smad3 (19) and Smad4 (20) expression vectors was performed using an electroporation kit (Amaxa Biosystems, Cologne, Germany). Cell growth was estimated by MTS assay, using a specific kit (Promega, Madison, WI), according to the manufacturer’s protocol.

Multiplex PCR and real-time PCR. Total RNA was prepared using a RNeasy™ mini kit (Qiagen). Reverse transcription (Invitrogen) was performed on genomic DNA-free RNA using oligo(dT) as primer. cDNAs are then used in multiplex PCR according to Qiagen recommendations. Real-time PCR was performed with either a Power SYBR Green mix or Taqman probes (mouse experiments) on an AB7300 apparatus (Applied Biosystems). The absolute copy number for each mRNA was normalized to the absolute cyclophilin A mRNA copy number. PCR primer sequences and conditions are available upon request.
**Western Blot analyses.** Cells were lysed with ice-cold lysis buffer (20 mM HEPES pH=7.9, 420 mM NaCl, 0.5% NP-40, 25% glycerol, 1.5 mM MgCl2 and 0.2 mM EDTA), rapidly frozen and thawed, rotated 30 min. at 4°C and centrifuged 30 min. 100 µg of total protein were separated by SDS-PAGE and blotted onto nitrocellulose membranes. After saturation with TBS+0.1% tween-20+5% dry milk, membranes were incubated with either anti-Gli2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or anti-actin (Zymed, San Francisco, CA) antibodies. Detection was performed using HRP-conjugated secondary antibodies (Santa Cruz) and revealed with ECL (Amersham Biosciences, Uppsala, Sweden).

**Transgenic mice.** To determine the capacity of TGF-β to induce Gli expression *in vivo*, we used a transgenic mouse model constitutively expressing TGF-β1 in the epidermis under the control of the keratin 5 promoter (K5-TGF-β1, (21), as well as an inducible gene-switch mouse model allowing inducible expression of TGF-β1 in the epidermis under the control of the progesterone receptor (22). To determine the relative implication of Smad2 and Smad3 in mediating TGF-β effects on Gli expression, K5-TGF-β1 mice were crossed with either Smad2+/- or Smad3+/- mice (23) and Gli expression was measured in compound heterozygote animals.
Results and Discussion

TGF-β activates Gli1 and Gli2 expression in various cell types: We first examined whether TGF-β has a direct effect on the expression of various components of the Hh signaling cascade. For this purpose, cultures of human neonatal dermal fibroblasts (NHDF, Fig. 1A), HaCaT keratinocytes (Fig. 1B), and MDA-MB-231 breast carcinoma cells (Fig. 1C) were incubated with TGF-β and RNA was extracted at various time points. Remarkable conservation of the modulation of Gli1 and Gli2 expression was observed in all cell-types tested. Specifically, rapid and persistent induction of Gli2 was observed in response to TGF-β, peaking at 2-8h, and remaining at levels significantly higher than their basal expression state up to 16-24h. On the other hand, delayed induction of Gli1 was observed, with a maximum around 48h after TGF-β addition. Similar patterns of Gli1 and Gli2 regulation by TGF-β were also identified in human adult primary skin fibroblasts; immortalized lung fibroblasts (WI-26 cell line) and keratinocytes (NCTC2544 cell line), as well as other human cancer cell lines, including pancreatic adenocarcinoma, glioblastoma and melanoma (not shown).

Western analysis of total Gli2 production in dermal fibroblasts showed undetectable expression levels in unstimulated cultures. Gli2 protein became detectable 6h after addition of TGF-β, and accumulated over the 48h of incubation (Fig. 1D). Given the high levels of Gli2 protein detected at the 48h time-point, it is possible that TGF-β may stabilize GLI2 protein, in addition to increasing gene expression.

Of note, Gli3 (see multiplex PCR panels), Ptc, and Smo (not shown) expression in response to TGF-β showed minimal variation.
Activation of Gli expression by TGF-β does not involve the Ptch/Smo axis: To determine whether TGF-β-induced Gli1 and Gli2 expression was dependent on the Ptch/Smo axis, the effect of exogenous Shh and TGF-β on Gli expression by human dermal fibroblasts was tested in the absence or presence of the Smo inhibitor cyclopamine, known to prevent Gli activation by Shh (24). As shown in Fig. 2A, both TGF-β and Shh strongly elevated Gli1 mRNA steady-state levels. As expected, cyclopamine abrogated Shh-induced Gli expression (lane 4 vs. lane 3), but had no effect on TGF-β-mediated Gli1 activation (lane 6 vs. lane 5). Furthermore, when added together to the culture medium, Shh and TGF-β exerted additive effects on both Gli2 and Gli1 expression (Fig. 2B). Under the same conditions, Ptch-1, a classic Shh target, was strongly induced by Shh, not by TGF-β (upper panel), suggesting that Ptch-1 modulation is not solely dependent on Gli expression. Interestingly, three distinct skin fibroblast strains derived from patients with Gorlin’s syndrome that carry heterozygote somatic loss-of-function mutations of the Ptch-1 gene (25), responded to TGF-β with significant upregulation of both Gli2 and Gli1 expression, with kinetics similar to those observed in non Gorlin-related cells. Specifically, all three Gorlin fibroblast strains exhibited a 4- to 11-fold transient elevation of Gli2 mRNA 4h after TGF-β stimulation Fig. 2C, left panel), followed by a 4- to 9-fold elevation of Gli1 mRNA steady-state levels 24h after addition of TGF-β (Fig. 2C, right panel).

Together, these results indicate that Shh and TGF-β are capable of inducing Gli expression via distinct mechanisms and that the mechanisms underlying TGF-β effects do not involve the Ptch/Smo axis.

Gli2 induction by TGF-β is a Smad3-dependent mechanism and mediates subsequent Gli1 activation: As a first attempt to elucidate the mechanisms by which TGF-β activates
Gli1 and Gli2 expression, the possible implication of de novo protein synthesis was determined. As shown in Fig. 3A, the protein synthesis inhibitor cycloheximide abrogated Gli1 induction by TGF-β, but had no inhibitory effect on Gli2 activation. Thus, the rapid elevation of Gli2 expression in response to TGF-β involves existing transactivators, while the delayed activation of Gli1 requires de novo synthesis of mediators.

Among the immediate transduction pathways activated by TGF-β, the most important and ubiquitous one is the Smad cascade (18), although evidence exists for activation of MAP kinases, SAPK/JNK, ERK, and p38/MAPK, and of the PI3-kinase/AKT pathways, downstream of the TGF-β receptors (26, 27). As a first approach to discriminate between all pathways, we tested the effects of pharmacologic inhibitors that specifically target the MAPK and PI3K pathways in parallel to a TβRI/ALK5 inhibitor on TGF-β-induced Gli2 expression.

As shown in Fig. 3B, neither the MAPK inhibitors SB203580, PD98059, and SP600125, nor the PI3K inhibitor LY294002, which all effectively blocked their respective target pathway in these experiments (not shown), affected TGF-β effect on Gli2 expression, while the TβRI/ALK5 inhibitor SB431542 fully abrogated the induction.

Next, we used the breast carcinoma cell line MDA-MB-468, which carries a large homozygous deletion within chromosome 18 encompassing the entire Smad4 gene (28), and exhibits deficient Smad-dependent transcription in response to TGF-β (19, 20). Gli2 expression in mock-transfected cells was barely detectable and was not elevated in response to TGF-β (Fig. 3C, lane 2 vs. lane 1). However, with Smad3/4 complementation, Gli2 induction by TGF-β was fully restored in these cells (lane 4 vs. lane 3), thus unequivocally implicating the Smad pathway in Gli2 activation by TGF-β.

Further evidence for the need for intact Smad signaling came from Smad3 knockdown experiments: transfection of specific Smad3 siRNAs into WI-26 fibroblasts prior to TGF-β stimulation markedly impaired the induction of Gli2 gene expression by TGF-β, while control
siRNAs did not (Fig. 3D). Similar observations were made in HaCaT keratinocytes (not shown).

Genetic approaches have demonstrated that Gli2 plays the preeminent role in the transcriptional response to Hh signaling, and is required for induction of Gli1 (5, 29). Since Gli2 induction by TGF-β precedes that of Gli1, we hypothesized that, similar to Hh signaling, late Gli1 induction by TGF-β may be dependent on early Gli2 activation. Consistent with this hypothesis, Gli2 knockdown with Gli2 siRNAs, which efficiently abrogated Gli2 induction by TGF-β (Fig. 3E, right panel), prevented Gli1 induction in WI-26 human lung fibroblasts (left panel). Similarly, over-expression of a dominant-negative mutant form of Gli2, mGli2-EN, which lacks transcriptional activity (30), also partially abrogated the induction of Gli1 by TGF-β (not shown).

Together with the fact that cyclopamine did not prevent Gli1 induction by TGF-β (see panel A), these results demonstrate a Shh/Ptch/Smo-independent, Smad3-dependent, activation of Gli2 in response to TGF-β, which leads to delayed induction of Gli1. Thus, TGF-β is capable of conveying signals that were initially thought to be almost exclusively dependent on Hh factors.

**TGF-β activates Gli1 and Gli2 expression in vivo in a Smad3-dependent manner:** To determine whether TGF-β was capable of modulating Gli expression in vivo, two distinct transgenic mouse models overexpressing TGF-β1 in the epidermis (see Materials and Methods) were examined. Quantitative RT-PCR indicated that Gli1 expression was 5- to 6-fold higher in the epidermis from both K5-TGF-β1 mice with sustained TGF-β1 transgene expression (21) and gene-switch-TGF-β1 mice with acute TGF-β1 transgene induction (22), compared to the epidermis of control animals, while Gli2 expression was 12- to 15-fold higher than in controls (Fig. 4A). Next, to determine whether Smad signaling was implicated
in Gli induction by TGF-β in vivo, Gli expression levels were measured in the epidermis of K5-TGF-β1 transgenic mice and of either K5-TGF-β1xSmad2+/- or K5-TGF-β1xSmad3+/- compound heterozygote mice. As shown in Fig. 4B, Smad3, not Smad2, heterozygocity suppressed high Gli expression in the skin of K5-TGFβ1 mice, consistent with the broad reduction in TGF-β target gene expression levels described previously (31). These data unequivocally demonstrate the implication of Smad3 in TGF-β-induced Gli2 and Gli1 expression in vivo, in accordance with the in vitro results described in this report that identify Smad3/4-dependent Gli activation by TGF-β in cell lines of both mesenchymal and epithelial origins, and consistent with the described role of Smad3 vs. Smad2 in mediating most transcriptional responses to TGF-β (17, 18).

Pharmacologic inhibition of TGF-β signaling inhibits the growth of cyclopamine-resistant pancreatic adenocarcinoma cell lines: There is ample evidence that Gli genes possess activities that are independent from Hh signaling. For example, Gli2 and Gli3 are widely expressed in the developing embryo, including in regions that are far from Shh production (32), and may be expressed downstream of FGF signaling (33). Also, mice expressing mutant forms of Gli2 or Gli3 exhibit defects that are likely not solely dependent on loss of Hh signaling (34). Thus, what is crucial for a given cell is the overall state of Gli expression and function, and Hh might just be one of several ways to regulate it (2). In this context, a growing number of reports indicates the potential therapeutic benefit of targeting either the TGF-β/Smad or Shh/Gli signaling pathways to counter the neoplastic process, consistent with their respective pro-oncogenic capacities. For example, cyclopamine and small-molecule Shh antagonists have shown efficacy in reducing tumor burden and inducing cancer cell apoptosis (13, 35, 36). Likewise, interfering with TGF-β signaling has shown promising results in preventing tumor development in a variety of tumor types (37-42). To
identify specific molecular signatures in a given tumor is critical to help determine the adequate therapeutic strategy (43). Interestingly, in a study involving numerous pancreatic carcinoma cell lines, inhibition of the Hh pathway by cyclopamine led to reduced proliferation and increased apoptosis only in a subset of cells. On the other hand, the authors identified another subset of cancer cells that was entirely resistant to cyclopamine, suggesting that their constitutive Hh activation, estimated as Gli1 or Ptch-1 expression, may not occur via Ptch/Smo (13). We thus tested the possibility that TGF-β/Smad signaling may contribute to constitutive Gli expression in the cyclopamine resistant PANC-1 cell line (13), and whether pharmacologic inhibition of the TGF-β pathway may inhibit their proliferation. As shown in Fig. 5A, PANC-1 cells showed significantly impaired growth when treated with the TβRI/ALK5 inhibitor SB431542, not with cyclopamine, accompanied with decreased basal Gli2 expression, as measured by quantitative RT-PCR (Fig. 5B), suggesting that autocrine TGF-β signaling may contribute to basal Gli2 expression. Similar results were obtained with the BxPC-3 cell line (not shown). Transfection of PANC-1 cells with Gli2 siRNA efficiently reduced basal Gli1 expression (Fig. 5C), PANC-1 cell growth (Fig. 5D), and strongly attenuated the anti-proliferative effect of SB431542 (Fig. 5D). Together, these data indicate that the growth inhibitory activity of SB431542 on PANC-1 cells is, at least in part, driven by a reduction in Gli expression.

**Conclusion:** We have identified TGF-β as a potent inducer of both Gli1 and Gli2 expression, independent from the Ptch/Smo axis. Most importantly, Gli2 induction is rapid, does not require de novo protein synthesis, and is a direct target of the Smad pathway. These results demonstrate unambiguously that TGF-β is capable of directly inducing signals that, until now, were thought to be exclusive mediators of Hh signaling, and open new venues for efficient therapeutic approaches against cancer progression.
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References


Legends to Figures

Figure 1
Effect of TGF-β on Gli1 and Gli2 mRNA levels. Subconfluent neonatal human dermal fibroblasts (NHDF, panel A), HaCaT keratinocytes (panel B), and MDA-MB-231 human breast carcinoma cells (panel C), were treated with TGF-β1 (5 ng/ml) for the indicated times. Expression of Gli1, Gli2 and Gli3 was estimated in parallel by multiplex PCR. The expression of the housekeeping gene GAPDH was used as a control. A representative ethidium bromide-stained agarose gel is shown for each cell type on the left side. Real-time PCR was then performed on the same samples to quantify the effect of TGF-β on Gli1 (gray squares) and Gli2 (solid triangles) mRNA steady-state levels. Results are shown as fold-induction above mRNA levels at time 0. Panel D, normal human dermal fibroblasts were incubated with TGF-β and total proteins were extracted at various time points. Gli2 protein content was detected with an anti-Gli2 antibody. An antibody directed against β-actin was used to verify equal protein content in each sample.

Figure 2
TGF-β effect on Gli expression does not require Shh signaling. (A), NHDF were pre-treated with cyclopamine (CPN 5 µM) for 30 min. and stimulated with either Shh (1.5 µg/ml) or TGF-β1 (5 ng/ml) for 24h. Patched-1 (PTCH-1), Gli1, and Gli2 expression were estimated by multiplex PCR (left panel). Gli1 expression was subsequently quantified by real-time PCR (right panel). (B), NHDF were treated with either Shh (1.5 µg/ml) and/or TGF-β1 (5 ng/ml) for 24 hours. Patched-1, Gli1 and Gli2 expression were estimated in parallel by multiplex PCR (left panel). The modulation of Gli1 and Gli2 expression was quantified by real-time PCR, as described in Materials and Methods. (C), Three distinct Gorlin patients’-derived...
fibroblast (GHDF) cultures (AS573, AS578, and AS587) were treated with TGF-β1 (5 ng/ml) for 4 or 24h. Gli2 (left panel) and Gli1 (right panel) expression was measured by Q-PCR.

**Figure 3**

*Gli2* is a direct TGF-β/Smad target gene and mediates *Gli1* activation by TGF-β. (A), NHDF were pre-treated with cycloheximide (CHX, 10µg/ml) for 30 min. prior to incubation with TGF-β. 24h later, *Gli1* and *Gli2* expression were estimated in parallel by multiplex PCR (left panel) and their expression was quantified by real-time PCR (right panel). (B), WI-26 fibroblast cultures were pre-treated with various pharmacologic inhibitors (SB431542: 5µM; SB203580: 25µM; PD98059: 50µM; SP600125 and LY294002: 20µM), as indicated, for 30 min. prior to stimulation with TGF-β. 4h later, *Gli2* expression was quantified by real-time PCR. (C), MDA-MB-468 breast carcinoma cells were transfected with either empty pcDNA3.1 or Smad3 and Smad4 expression vectors. 40h later, cells were stimulated for 4h with TGF-β. *Gli2*, *Smad3* and *Smad4* expression levels were simultaneously estimated by multiplex PCR. (D), WI-26 cultures were transfected with Smad3 siRNA, serum starved for 16h, then stimulated for 4 hours with TGF-β. *Gli2* and *Smad3* expression were quantified by real-time PCR. (E), WI-26 cultures were transfected with *Gli2* siRNA, serum starved for 16h, then stimulated with TGF-β for 48h, following which *Gli1* and *Gli2* expression levels were quantified by real time PCR. (B, D, E): without (☐), with (■) TGF-β.

**Figure 4**

TGF-β1 activates *Gli1* and *Gli2* expression in vivo in a Smad3-dependent manner. (A), Total RNA was extracted from the epidermis of control, gene-switch-*TGF-β1*, and K5-*TGF-β1* transgenic mice (see Materials and Methods). *Gli1* and *Gli2* mRNA steady-state levels were determined by real-time PCR. (B), K5-*TGF-β1* transgenic mice were crossed with either
Smad2+/- or Smad3+/- heterozygotes. Gli1 and Gli2 expression levels in the epidermis of control and K5-TGF-β1 animals were compared to that in the skin of compound heterozygote animals. In both panels, the results are the mean +/- sem of values from 4 animals in each group.

**Figure 5**

Pharmacologic inhibition of TGF-β signaling inhibits the growth of the cyclopamine-resistant pancreatic adenocarcinoma cell line PANC-1. (A), PANC-1 cell cultures in logarithmic growth phase were incubated in the absence or presence of either cyclopamine or SB431542 (both at 5µM), alone or in combination. Medium containing fresh inhibitors was changed every other day and incubations continued over a 7-day period. Cell proliferation was measured using a commercial MTS assay. Results expressed as a percentage of growth relative to untreated cultures at the same time-point are the mean +/- sem of triplicate culture dishes. (B), Sub-confluent PANC-1 cell cultures were incubated in the absence or presence of either cyclopamine or SB431542, alone or in combination for 48h. RNA was extracted and Gli2 expression was determined by quantitative RT-PCR. Expression of GAPDH in each sample was used for normalization. (C), PANC-1 cells were transfected with Gli2 siRNA in medium containing 5% serum. 48h later, Gli2 and Gli1 expression levels were quantified by real time PCR. (D), PANC-1 cells were transfected with either control or Gli2 siRNA. 72h later, cells were treated without or with SB431542 (5µM) and cell growth was examined by MTS assay after a 3-day period.
Figure 1

A. NHDF

B. HaCaT

C. MDA-MB-231

D. NHDF

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Figure 4

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