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Cooperative Hedgehog-EGFR signaling

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1. ABSTRACT

It has been known for many years that cooperative interactions between oncogenes (e.g. RAS, MYC, BCL2) can fuel cancer growth (1-5), but the restricted druggability of many of those interacting cancer genes has hampered translation of combined targeting to medical cancer therapy. The identification and characterization of cooperative cancer signaling pathways amenable to medical therapy is therefore a crucial step towards the establishment of efficient targeted combination treatments urgently needed to improve cancer therapy. Here we review recent findings of our group and colleagues on the molecular mechanisms of cooperative Hedgehog/GLI and Epidermal Growth Factor Receptor (EGFR) signaling, two clinically relevant oncogenic pathways involved in the development of many human malignancies. We also discuss the possible implications of these findings for the design of a therapeutic regimen relying on combined targeting of key effectors of both pathways.

2. INTRODUCTION

Cancer is a dreadful and frequently incurable disease that accounts for more than 7 million deaths per year worldwide. Although cancer research has made impressive progress to better understand and treat malignant diseases, there is still an unmet high medical need to transfer detailed molecular knowledge into more efficient cancer therapies. Cancer cells harbor a considerable number of genetic and epigenetic alterations though only a relatively small fraction of these alterations referred to as driver mutations is considered to account for malignant transformation and cancer growth (6-10). The identification of driver mutations for all cancer entities is critical to decipher the etiology of malignant development and eventually essential for the design of efficient therapies specifically tailored to a given cancer type as well as to the genetic make-up of the patient´s cancer cells. However, this is an extremely difficult and laborious task as it requires thorough functional assays of all possible oncogenic and

tumor suppressing alleles (6). Also, the well documented cooperative interaction of oncogenes and tumor suppressor genes can mask or complicate the identification of mutations responsible for malignant transformation as such events alter the cellular phenotype only if they occur in combination rather than as single alterations.

A detailed molecular understanding of druggable cooperative oncogenic signaling pathways would provide a rationale for the development of novel therapeutic strategies selectively targeting synergistic interactions of such signals within complex gene networks. The cooperative nature of oncogenic signals also suggests that malignant transformation heavily relies on synergistic modulation of downstream genes and signaling circuitries. This has been underpinned by the finding that the synergistic target gene profile regulated by cooperative oncogenic mutations such as RAS activation and TP53 deletion is highly enriched for genes essential for the determination of the malignant phenotype of a cell. Studying cooperative oncogenic signals therefore provides a rationale for the identification of additional therapeutic targets downstream of the initial genetic events that may not be amenable for therapeutic intervention and also allows development for multi-hit targeted combination therapy (11).

In this review we will concentrate on the cooperative interaction between the EGFR and the HH/GLI pathway, two druggable oncogenic signals with high clinical relevance in a number of human malignancies. We aim to provide a summary of recent findings on the synergistic cross-talk between these pathways and downstream signal cascades and discuss possible therapeutic implications derived from in-depth analysis of such molecular interactions in cancer cells.

3. OVERVIEW ON MECHANISMS OF HEDGEHOG/GLI SIGNAL TRANSDUCTION

The Hedgehog pathway was initially identified in *Drosophila melanogaster* (12) and has been shown to be highly conserved between invertebrates and mammalian species, although some substantial differences of how the signal is transduced and coordinated at the cell surface exist (13, 14). HH/GLI signaling is a complex process involving numerous regulatory steps and proteins that control a variety of developmental decisions in vertebrates and invertebrates. In the following, only a brief and condensed introduction into the key steps and components of vertebrate HH/GLI signaling is presented.

In vertebrates, at least three Hedgehog homologues have been identified referred to as Sonic Hedgehog (SHH), Indian Hedgehog (IHH) and Desert Hedgehog DHH), of which SHH is the most intensely studied. To initiate the Hedgehog signaling cascade, HH–ligand binds to its receptor, the 12-pass transmembrane protein Patched (PTCH), which is localized in the so called ‘primary cilium’, a non-motile structure protruding from the cell surface that functions as a signal sensing and coordination center for the HH pathway. Unliganded PTCH located at the base of the cilium inhibits pathway activation by preventing the seven-transmembrane protein Smoothened (SMO) to enter the cilium, a mechanism that is not yet fully understood (15, 16). This results in the formation of repressor forms of the GLI zinc finger transcription factor GLI3 – and to some extent of GLI2 – which repress HH target gene expression (17). HH pathway activation involves binding of HH to PTCH, thereby abolishing its repressive function on SMO. This allows SMO to enter the cilium and promote the formation of GLI activator transcription factor forms (GLI-A). GLI2/3-A forms then translocate to the nucleus and activate HH/GLI target genes including the third member of the GLI family, GLI1, which further increases the level of GLI activator (18-24).

3.1. HH/GLI signaling and cancer

More than 20 years after its discovery in the fruit fly, the HH/GLI pathway has now become one of the key signaling pathways in cancer research. HH/GLI signaling has been implicated in a number of human malignancies, including cancers of the brain, skin, gastrointestinal tract and hematopoietic system (21, 25-29). Uncontrolled HH/GLI pathway activation is a common feature of many cancers with cell-autonomous, autocrine and paracrine signal transduction settings (30). HH/GLI pathway activation can be triggered by mutational inactivation of pathway repressor proteins such as PTCH or SUFU, by mutations leading to constitutive activation of the HH effector SMO or by gene amplification of the GLI transcription factors GLI1 or GLI2 (31-36). This results in ligand-independent, cell-autonomous activation of HH/GLI signaling in cancer cells, a setting that applies to Gorlin-type tumors such as basal cell carcinoma, medulloblastoma or rhabdomyosarcoma (37, 38). The mode of HH signal transduction in the class of ligand-dependent cancers such gastrointestinal cancers is different as tumor cell-derived HH ligands have been shown to activate paracrine HH signaling with the adjacent tumor stroma - rather than the tumor itself - being the responsive compartment (39). By contrast, malignant cells of the hematopoietic system display cell-autonomous intrinsic HH pathway activation most likely through paracrine stimulation by HH ligands secreted by the adjacent tumor stroma (40). This mode of signaling as well as autocrine HH pathway activation also applies to the tumor-initiating cancer stem cell population of solid cancers and hematopoietic malignancies (41-44).

Based on the well documented etiologic role of HH signaling in cancers, targeting the pathway is considered a promising therapeutic strategy. In fact, a number of small molecule inhibitors of the essential pathway effector SMO have been identified as potent anti-cancer agents in preclinical models (reviewed in (30)). The therapeutic benefit of some of these SMO antagonists are currently evaluated in a number of clinical trials with first promising results for the treatment of advanced or metastatic basal cell carcinoma and medulloblastoma (45, 46).

Although SMO inhibition has already proven a valid therapeutic target in a selection of cancer patients, the

ongoing and additional phase II clinical trials will finally show the benefit of this regimen. On this long road, several obstacles will have to be by-passed and problems such as development of drug resistance and intricate interactions with other oncogenic signaling pathways taken into account. For instance, treatment of a metastatic medulloblastoma patient with the SMO inhibitor GDC-0449 led to a dramatic improvement of the patient´s condition after only two months of therapy. However, after three months the same patient relapsed due to development of drug resistance, which arose in response to a SMO mutation that abrogates binding of GDC-0449 to SMO (45, 47). Similarly, prolonged SMO antagonist treatment of HH-driven medulloblastoma mice led to drug resistance due to amplification of Gli2, rare SMO mutations and/or a parallel increase of PI3K/AKT/mTOR signaling that circumvented and compensated for the HH pathway blockade, respectively. Of note, treatment with a combination of SMO inhibitor and PI3K-mTOR antagonists delayed resistance development, providing a strong argument for rationale-based targeted combination therapy of selected HH-associated cancers (48).

The therapeutic success of HH/SMO inhibition will eventually also depend on the proper combination of therapeutic drugs targeting signaling molecules or pathways that have been shown to positively interact with and enhance HH signaling, in line with the current concept of highly inter-connected, cooperative signaling networks in cancer cells. The list of oncogenic signals interacting with HH/GLI signaling is constantly growing and includes a number of well-known druggable effectors such as PI3K-AKT, RAS, MEK/ERK or EGFR (49-51).

In the following we will concentrate on the synergistic interaction of the HH/GLI and EGFR signal transduction pathway and review recent findings of our group and colleagues on the mechanism of signal cooperation and its possible exploitation for the design of targeted cancer therapy based on a combined HH/GLI-EGFR inhibitor regimen.

4. SIGNAL TRANSDUCTION BY THE EPIDERMAL GROWTH FACTOR RECEPTOR FAMILY

The Epidermal Growth Factor Receptor (EGFR) family of receptor tyrosine kinases (RTKs) comprises four members, EGFR (or ErbB1/HER1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4), which all show conserved structural features and are expressed in numerous epithelial, mesenchymal and neuronal cell types, where they regulate a broad spectrum of biological processes key amongst which are proliferation and survival (52-55). Eleven different ligands are known that bind the extracellular domains of HER1, HER3 and HER4 with different and partly redundant specificity. EGF for example binds exclusively to EGFR while the Neuregulins 1 and 2 bind to HER3 and HER4. HER2 lacks a soluble ligand but plays an important role as heterodimerization partner for other family members. Ligand binding initiates the signaling cascade by inducing the formation of functional homo- or heterodimers, followed by the activation of the intrinsic tyrosine kinase domain. As a consequence, the C-terminal cytoplasmic tails of ErbB receptor dimers become phosphorylated at defined tyrosine residues that then act as context-dependent docking sites for adaptor or effector proteins of least five different signaling pathways: the mitogen-activated protein kinase (MAPK) pathway, phospholipase C, phosphatidylinositol 3-kinase (PI3K)/Akt pathway, signal transducer and activator of transcription (STAT) pathway and the SRC/FAK pathway (55).

Aberrant activation of EGFR signaling is a hallmark of many carcinomas. Mutational activation of EGFR proteins, overexpression of EGFR family members or of their cognate ligands has been identified as etiologic step in the development and growth of a number of malignancies. Hyperactivation of EGFR signaling in cancer cells is frequently accompanied by the stimulation of the intracellular RAS/RAF/MEK/ERK and PI3K/AKT signaling cascades which are considered the major proliferation and pro-survival signals, respectively (56). In agreement with its crucial role in the control of cancer cell proliferation, survival and angiogenesis, targeted inhibition of the EGFR pathway with small molecule inhibitors or therapeutic antibodies offers a benefit to many cancer patients as has been demonstrated for instance for breast cancer patients or lung cancer patients with activating mutations in the EGFR (57-60).

4.1. Cooperative HH/GLI-EGFR signal transduction

First evidence for an interaction of the vertebrate HH/GLI and EGFR signaling pathway has come from *in vitro* studies of neocortical stem cells showing that Shh and EGF cooperate in the stimulation of cell proliferation (61, 62). In addition, over-expression of SHH in a human keratinocytes cell line grown in organotypic cultures led to EGFR activation and increased levels of JUN and MMP9, thereby enhancing the invasive phenotype of the keratinocytes (63).

In a screen for oncogenic signal transduction pathways interacting with HH/GLI signaling our own group discovered that combined activation of EGFR signaling and GLI expression in human epidermal cells results in the selective and synergistic activation of a subset of direct GLI target genes. We identified a panel of genes whose expression is activated by simultaneous GLI-EGFR activation but not – or only to low levels - in response to single pathway stimulation. Of note, some of these cooperation response genes (CRG) like IL1R2, S100A9 and JAG2 have functional GLI binding sites in their promoter region, suggesting convergence of cooperative HH/GLI – EGFR signaling at the level of the *cis*-regulatory region of selected HH target genes (64). In a follow up-study, Schnidar *et al.* (2009) demonstrated that combined activation of HH/GLI and EGFR signaling not only modifies the GLI target gene expression profile but also leads to synergistic oncogenic transformation. While neither GLI activator expression nor EGFR stimulation alone was sufficient to induce anchorage-independent growth in 3D cultures - a hallmark of cancer cells that correlates well with *in vivo* tumorigenicity - simultaneous activation of both pathways resulted in a transformed

phenotype with clonogenic growth under non-adherent conditions. Of note, this cooperative transformation effect could be observed in various cell types of different species including human keratinocytes, rat kidney epithelial cells and mouse fibroblasts pointing to a more common mechanism of cooperative oncogenic transformation by concomitant HH-EGFR signaling in various cancer types (65).

4.2. Intracellular convergence of cooperative HH/GLI – EGFR signaling via RAS/RAF/MEK/ERK

Activation of the PI3K/AKT and MAP kinase cascade is considered the major intracellular signaling event in response to EGFR stimulation (see above). Both pathways have been shown to affect the activity of GLI transcription factors, suggesting that activation of EGFR may directly impinge on GLI regulation. For instance, the nuclear localization of GLI1 can be enhanced by expression of dominant active AKT, while blocking AKT function results in an accumulation of GLI1 in the cytoplasm. Also, in melanoma, glioma and prostate cancer cells GLI1 expression itself is decreased if PI3K/AKT signaling is inactivated and synergistic anti-proliferative effects could be detected in response to combined inhibition of AKT and SMO (66). Another study has revealed that PI3K/AKT negatively regulates the degradation of GLI2 by interfering with PKA/GSK3beta mediated phosphorylation of GLI2, which targets the protein to proteasome-mediated degradation (67). This cooperation of PI3K/AKT and HH/GLI signaling is supported by studies in zebrafish showing that co-expression of the zebrafish Smoothened in combination with constitutively active Akt1 directs tumor formation (68).

However, in the case of HH/GLI-EGFR cooperativity, several lines of evidence argue against a crucial role of EGFR-regulated PI3K/AKT activation in the integration with HH/GLI signaling. Both, genetic and pharmacologic inhibition of PI3K/AKT does not affect the synergistic regulation of HH-EGFR CRG expression nor the oncogenic transformation phenotype induced by combined HH/GLI-EGFR activation. Also, co-expression of dominant active AKT and GLI1 in non-tumorigenic human keratinocytes fails to enhance the tumorigenicity of the cells (65). This is in agreement with the observation that unlike wild-type EGFR, constitutively active, mutant EGFR variants that preferentially activate the PI3K/AKT cascade do not synergize with GLI in oncogenic transformation (65, 69).

In agreement with the requirement of RAS/RAF/MEK/ERK signaling downstream of EGFR, only co-expression of GLI1 and dominant active MEK induces tumor growth *in vivo*, as opposed to co-expression of GLI1 and dominant active AKT (65). Although GLI proteins have been proposed as putative substrates of MAP kinases (70-72), we could not find any evidence for direct phosphorylation of GLIs in response to EGFR/MEK/ERK signaling (64). Like PI3K/AKT, RAS/RAF/MEK/ERK activation has also been shown to increase the nuclear localization and transcriptional activity of GLI, but as our initial studies were done with nuclear export sequence mutants that constitutively localize to the nucleus, increased nuclear localization of GLI in response to EGFR/RAF/MEK/ERK signaling is unlikely to account for the synergistic CRG activation profile observed in our system (64, 65).

4.3. HH/GLI-EGFR signal integration in the nucleus via cooperation of GLI and JUN/AP-1

How is the synergistic signaling system of HH/GLI and EGFR integrated and how does cooperativity between the pathways lead to selective activation of cooperation response genes (CRG)? Analysis of the *cis*-regulatory region of some HH-EGFR CRG revealed the presence of functional GLI binding sites, suggesting convergence of both pathways at the level of the promoter of selected HH/GLI target genes. As the synergistic transcriptional activation by combined HH/GLI and EGFR pathway activation applies only to a subset of direct GLI targets but not to canonical HH targets such as PTCH or BCL2, simultaneous activation of GLI and EGFR signaling is likely to recruit additional cooperative transcriptional regulators to the promoters of cooperation response genes (64).

Transcriptional regulation in response to EGFR signaling involves members of the ETS, STAT and AP-1 family (52, 73). As some CRG promoters such as IL1R2, JAG2 and S100A9 harbor binding sites for both GLI and JUN/AP-1 factors, it is likely that at least some HH-EGFR CRG are synergistically regulated by combined binding of GLI and JUN/AP-1 transcription factors to their promoters. In agreement with this signal integration model, EGFR signaling via RAF/MEK/ERK leads to phosphorylation and activation of JUN in human keratinocytes, and interference with JUN function impairs HH-EGFR CRG expression and synergistic oncogenic transformation by cooperative HH-EGFR signaling (65). As JUN expression itself has been shown to be directly enhanced by GLI (74), we further propose the existence of an EGFR-GLI regulated positive feed-forward loop accounting for sustained and enhanced levels of activated JUN/AP-1 transcription factors. In such a model, EGFR-MEK/ERK signaling induces phosphorylation of JUN, which together with GLI activator forms binds to cooperation response genes to activate CRG transcription and drive oncogenic transformation in a combinatorial mode (Figure 1). Whether integration of EGFR-HH/GLI signaling involves additional EGFR-regulated transcription factors and if this model holds true for *in vivo* carcinogenesis remains to be tested in future studies.

4.4. Combined inhibition of HH/GLI-EGFR signaling as anti-cancer therapy

Despite recent reports on the progress in cancer therapy, the disease is still considered incurable in many cases and finding the Achilles heel(s) of cancer cells is a major challenge for today´s oncology research. Perhaps the most promising approach to significantly improve the patients survival is to attack cancer cells from multiple sides using defined combinations of targeted anti-cancer drugs alone or along with chemotherapy, surgery and/or radiation therapy. The identification of novel efficacious

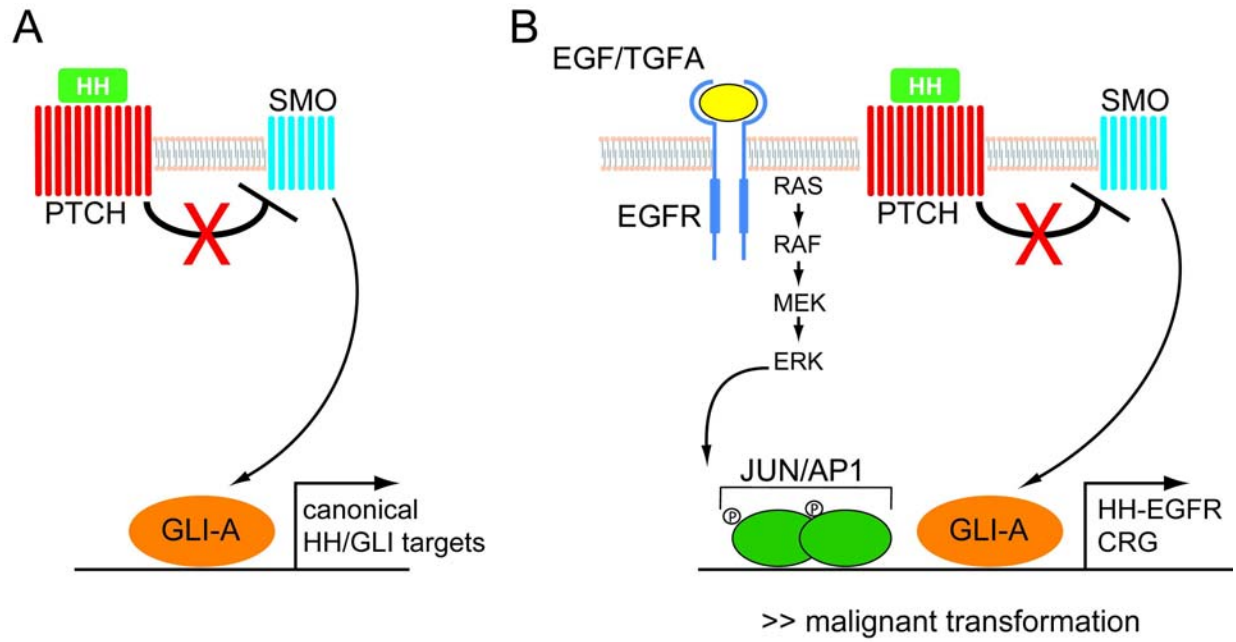

Figure 1. Model of cooperative HH/GLI-EGFR signal integration in malignant transformation. A) Activation of a canonical GLI target gene expression profile in the absence of parallel EGFR signaling. B) Simultaneous stimulation of HH/GLI and EGFR signaling results in the synergistic activation of HH/GLI-EGFR cooperation response genes (CRG) thereby promoting malignant transformation. HH/GLI-EGFR CRG are transcriptionally controlled by cooperative interactions of the GLI activator forms (GLI-A) and JUN/AP1 transcription factor complexes at the promoters of HH/GLI-EGFR CRG. Activation of JUN/AP1 transcriptional regulators (indicated by phosphorylation symbols) involves EGFR-dependent activation of the RAS/RAF/MEK/ERK cascade.

combinations of targeted cancer drugs will therefore be key to the success for such multimodal treatment strategies. The discovery of druggable, cooperative oncogenic pathways such as EGFR and HH/GLI may be a first step towards the design of a novel, rationale based combo treatment. Several *in vitro* studies suggest that the combination of specific EGFR and HH signaling inhibitors may provide a therapeutic benefit. For instance, treatment of metastatic prostate cancer cell lines or putative prostate cancer stem cells with a combination of the selective EGFR inhibitor gefitinib, the SMO antagonist cyclopamine and/or the chemotherapeutic drug docetaxel inhibits cell growth, induces apoptosis and/or interferes with invasiveness (75, 76). Our own studies have shown that combined inhibition of EGFR and HH/GLI signaling in mouse BCC cell lines efficiently interfered with cell growth *in vitro*, as did combined inhibition of GLI and JUN function (65). Since both the EGFR and HH/GLI pathway have been implicated in the pathogenesis of a considerable number of human cancer entities such as brain, skin, breast cancer, colon cancer, pancreatic, breast, colon and liver it is tempting to speculate that cooperative interactions occur also in these malignancies and that combinatorial targeting may therefore provide a therapeutic benefit to a considerable number of cancer patients.

5. PERSPECTIVE

The work of many laboratories during the past few years has unraveled a tremendous complexity of regulatory networks and signal interactions impinging on and regulating the activity of the Hedgehog pathway in cancer cells. Whether the detailed knowledge of these intricate signal interactions can be translated into therapeutic regimens in the clinic is unclear at present. As for the cooperative interaction of HH/GLI and EGFR signaling in human cancer cells, several key questions remain to be addressed. In particular, the *in vivo* relevance of the findings reviewed in this article need to be demonstrated in appropriate preclinical assays, for instance by genetic and pharmacologic inhibition of EGFR signaling in mouse models of HH-driven cancer. Also some of the apparently conflicting reports about the paracrine or autocrine mode of the HH/GLI pathway and the identity of the HH signal receiving cell type in human cancers need to be reconciled with the etiologic role of EGFR signaling in cancer cells. For instance, in pancreatic and colon cancer cells, canonical Hedgehog signaling is received by stromal cells rather than tumor cells (39, 77). Tumor cells are thought to produce and secrete high levels of HH ligands which then induce HH signaling in adjacent stromal cells in a paracrine mode of action. By contrast, $CD133^+$ tumor-initiating cells of colon cancer patients are susceptible to SMO inhibition and display cell-autonomous requirement of functional HH signaling (41). Therefore, it needs to be carefully addressed whether HH/GLI and EGFR signaling co-occur in cancer cells and whether co-activation of both pathways is established in the tumor bulk or in a subpopulation of tumor-initiating cancer cells.

In addition and in light of recent reports on SMO-independent regulation of GLI function (78), the requirement of SMO for the induction of GLI activator forms in cancer cells needs to be tested rigorously, as this will to a large extent determine the therapeutic efficacy of SMO inhibitors in combination with clinically approved anti-EGFR drugs.

In this context it is noteworthy that the therapeutic efficacy of simultaneous SMO and EGFR targeting by combined administration of GDC-0449 and erlotinib, a selective EGFR tyrosine kinase inhibitor, is currently evaluated in a clinical trial for the treatment of metastatic pancreatic cancer (source http://clinicaltrials.gov). Although the rationale of this study is mainly based on the observation that inhibition of paracrine HH/SMO signaling depletes the tumor stroma and thereby increases drug delivery to the tumor cells (79), it is well conceivable that the same treatment may also target the HH/GLI and EGFR pathway in cancer cells such as the tumor initiating subpopulation, which may depend on HH/GLI and EGFR signaling (80, 81).

More than twenty years after the discovery of the HH pathway in flies, we now have entered an exciting era of HH research, where the therapeutic benefit of HH antagonists developed by different pharmaceutical companies is evaluated in more than 25 clinical studies. The outcome of these trials will have a significant impact on the design of future cancer therapies involving HH/SMO inhibition and possibly also highlight the requirement for additional targeted combination treatments including protocols based on concomitant HH/GLI and EGFR inhibition.

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