Tumor-associated EGFR over-expression specifically activates Stat3 and Smad7 resulting in desensitization of TGF-β signaling

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The abbreviations used are: EGFR, epidermal growth factor receptor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FBS, foetal bovine serum; TGF-β, Transforming Growth Factor-beta; Stat3, Signal Transducer and Activator of Transcription 3; Smad, Sma- and Mad-related protein.

Abstract

Transforming Growth Factor-β (TGF-β) and Epidermal Growth Factor (EGF) signaling pathways are both independently implicated as key regulators in tumor formation and progression. Here, we demonstrate that activation of the tumor-associated and over-expressed EGFR desensitizes TGF-β signaling and its cytostatic regulation through specific Stat3 activation and Smad7 induction. In normal and tumor human cell lines, reduction of TGF-β-mediated Smad2 phosphorylation, nuclear translocation and Smad3 target gene activation were observed where EGFR is over-expressed, but not in cells which expressed EGFR at normal levels. The EGFR downstream signaling molecules phosphatidyinositol-3 Kinase (PI3K) or mitogen-activated protein kinase/ERK kinase (MEK) are not responsible for the down-regulation of TGF-β signaling since blockade of them by specific pharmacological inhibitors LY294002 and U0126 had little effects on the sensitivity of TGF-β signaling. We identified Stat3 as a signaling molecule activated specifically and persistently by over-expressed EGFR, but not by normal levels. Importantly, Stat3 is responsible for the reduced TGF-β sensitivity, since its knockdown by siRNA restored TGF-β signaling sensitivity. Furthermore, over-expressed EGFR, through Stat3 activates Smad7 promoter activity, increasing its protein levels, which is a negative regulator of TGF-β signaling. Consequently, cells were re-sensitized to TGF-β when Smad7 expression was reduced using siRNA. Therefore we establish a novel EGFR-Stat3-Smad7-TGF-β signaling molecular axis where tumor-associated over-expression of EGFR in epithelial cells results in hyperactivation of Stat3, which activates Smad7 expression, compromising the TGF-β's cytostatic regulation of epithelium and consequent tumor formation.

Introduction

Growth factor and cytokine signaling networks control many aspects of cell behaviour such as proliferation, survival, migration, invasive capabilities, transformation and differentiation. In normal cells, these complex signaling pathways are tightly regulated. Alterations of these signals are often found to cause, directly or indirectly, tumor formation. Transforming Growth Factor-β (**TGF-β)** and Epidermal Growth Factor (**EGF**) signaling pathways are both independently implicated as key regulators in tumor formation and as such they are potential therapeutic targets[1-4].

Ever since its discovery, EGFR has been intimately associated with cancer. Indeed, the cDNA of EGF receptor was first cloned from the human A431 carcinoma cell line, which over expresses the receptor protein[5-8]. Subsequently, the involvement of the EGFR in many human cancers has been established in cancers of the head&neck (90%), brain (30%), breast (30-50%), bladder (30-90%), stomach (30-70%), lung (45%), ovarian (30-80%) and prostate (10%)[9,10]. A number of studies further demonstrated that overexpression of EGFR or its tumor associated mutant forms resulted in tumor transformation *in vitro* and enhanced tumor growth *in vivo*, suggesting a causal effect of the elevation of the EGFR expression levels in carcinogenesis[11]. That role of EGFR in carcinogenesis led to the development and evaluation of EGFR blocking agents for cancer treatment[1]. Two EGFR-targeted approaches have been explored: one using monoclonal antibodies (mAbs) targeting its extracellular domain, and the other using small-molecular tyrosine kinase inhibitors (TKIs) targeting its intracellular tyrosine kinase[1,12,13]. A combination of the two or with chemotherapeutic treatment has also been evaluated[1]. The successful development of EGFR-specific TKIs gave rise to high hope that EGFR-blocking reagents could be the next generation of “magic bullets” in treating human cancers[14].

More than 20 phase II and III clinical trials targeting EGFR have been conducted, some are still active, on many cancer types, including cancers of the head&neck, colorectal, glioma, prostate, NSCL(non-small cell lung) and other types of cancers, with current trials focusing on NSCL cancers[1,15,16]. However, the patient response rate varied greatly from almost no response to over 50%, while the improvement on overall patient survival is uncertain[1,17]. While monotherapy targeting EGFR delivered some responses, early trials did not show improvement in combination therapy[1,17]. The reasons for their apparent lack of benefit when used in combination therapy are unclear. It is suspected that different administration schedules may be required[21]. Further research is necessary to establish their mechanism of action. Identification of accurate biomarkers may be needed to identify appropriate patients[1,18].

In normal cells, EGFR is expressed at relatively low levels (~10^4 receptors/cell). Its activation is controlled by ligand binding and dimerization/oligomerization[19]. Two main downstream pathways are activated, namely Ras-MAPK and PI3K-Akt[2]. Other pathways may also be activated, including Src, PLCγ and Stats[2]. The activation of Stats is identified, but has not been taken seriously as physiologically relevant mediators of EGFR biology. In EGFR-driven tumors, the receptor is normally expressed at higher levels (~10^6 receptors/cell). Although the many structures of the EGFR family, either with or without ligand complexing have been solved recently[20,21], we still do not fully understand how exactly the receptors are activated. Even more surprisingly and despite intensive scientific and clinical research, we are yet to identify which downstream molecules are specifically activated by tumor-associated and overexpressed EGFR and are responsible for EGFR's tumorigenic function. There is an urgent medical need to have this question answered.

TGF-β regulates a wide range of cellular processes including cell proliferation, differentiation, migration, organization and death[3]. As one of the most potent inhibitors of normal cell growth, the loss of growth inhibitory responses to TGF-β is often observed in

cancer cells[22,23]. It is widely accepted that TGF-β is a tumor suppressor, given the frequent occurrence of many types of tumors in mice with disruption of TGF-β or its signaling components by gene targeting and many types of human cancers containing loss-of-function mutation of TGF-β signaling components[3,24].

Biological responses to TGF-β are mediated mainly by the type I (TβRI) and II (TβRII) transmembrane cell surface receptors[4,25] which contain cytoplasmic domains with serine/threonine kinase activity. TGF-β ligands bind TβRI and TβRII thereby triggering phosphorylation and activation of TβRI. The activated ligand-receptor complex then binds and phosphorylates through TβRI the intracellular signaling molecules Smad2 and Smad3[4,25,26]. Once phosphorylated these regulatory Smads (R-Smad) form complexes with Smad4 (also called DPC4 for deleted in pancreatic carcinoma locus 4) and translocates into the nucleus. In the nucleus, they associate with transcription factors to form transcriptionally active DNA complexes[4,25,26].

TGF-β signaling can be negatively regulated at multiple levels in and out side the target cells[4]: secreted molecules such as decorin binds directly to TGF-β ligands and neutralize their biological activity; the transmembrane protein BAMB1 sequesters ligand from binding to TβRI; FKBP12 blocks receptor phosphorylation; the E3 ubiquitin ligase Smurf1 degrades R-Smads and TβR following binding to Smad7, while Smad7 directly competes with Smad2/3 for binding to TβRI.

The tight regulation of TGF-β signaling pathway at every step is critical in homeostasis, since any perturbation of the pathway *in vivo* appear to result in cancer forming in mice. Deletion of one copy of *Smad4* or *TGF-β1* gene resulted in gastric tumor formation in mice[27,28] as well as mice lacking the gene encoding the *RUNX3* transcription factor[29]. Intriguingly, the transcription factor RUNX3 is a target gene for TGF-β signaling and the

gastric epithelium of *RUNX3*$^{-/-}$ mice is desensitized to TGF-β-dependent growth suppression. Consequently, the mice also gave rise to gastric tumor formation[29]. Perturbation of TGF-β signaling by expressing a dominant negative form of *TβRII (DN-TβRII)*[30] or the negative regulator *Smad7*[31] as a transgene in mice also led to various forms of tumor formation. It remains to be answered whether the TGF-β signaling pathway is so fundamental in maintaining homeostasis that not only its direct disruptions, but also other oncogenic signals acting through the impairment of TGF-β signaling, leads to tumor formation.

Stat signaling pathways were originally delineated in the context of normal cytokine receptors such as interferon (IFN) and interleukin-6 (IL-6) receptors[32,33]. Evidence for a role of Stat3 in tumor transformation was provided by a constitutively activated mutant form, Stat3C, which was found to transform fibroblasts in culture, allowing them to form tumors in mice[34]. The first direct links between Stat3 and human cancer came from the findings that constitutive Stat3 activity is required for the growth of head&neck cancer cells and multiple-myeloma cells[35,36]. Subsequently, Stat3 activation has been detected at high frequency in diverse human cancer cell lines and tissues of blood, breast, head&neck, skin, lung and prostate[6].

We have recently discovered[37] that hyperactivation of Stat3 causes 100% penetration of gastric tumor formation in knock-in mice carrying an artificial mutant form of *gp130*^{Y757F}, (which results in sustained hyperactivation of Stat3), while the knock-in mice with a monoallelic deletion of *Stat3* are almost completely free of the tumors. Importantly, we have found that hyperactivation of Stat3 desensitizes TGF-β signaling, releasing cells from its cytostatic regulation, therefore allowing tumor formation. Subsequently, we identified the TGF-β signaling negative regulator Smad7 as a Stat3 transcriptional target. Identification of up-regulation of Smad7 by Stat3 provided a direct molecular link between the hyperactivation of Stat3 and the desensitization of TGF-β signaling in gastro tumorigenesis. This link is also

applicable to human, since in human gastric cancers, the increase of Stat3 activity occurs concomitantly with elevated Smad7 expression[37]. While Stat3 activation is observed in many types of tumors and increasingly, is becoming an anti-tumor target, there is no genetic evidence of its oncogenic mutation.

Here we establish a novel EGFR-Stat3-Smad7-TGF-β signaling molecular axis where tumor-associated over-expression of EGFR in epithelial cells results in specifically the sustained hyperactivation of Stat3, which induces Smad7 expression, compromising the TGF-β's cytostatic regulation of epithelium and consequent tumor formation.

Results

Over-expression of EGFR inhibits TGF-β signaling. A previous study showing that aberrant IL-6/gp130 signaling caused a desensitization in TGF-β response[37], led us to hypothesize that EGFR signaling could also mediate a similar outcome. Five human cell lines (A431, HN5, 293T, 293T-EGFR and A549 cells) with varying EGFR expression levels were used (Fig. 1a), to determine whether EGFR activation effected TGF-β signaling. The Smad3 luciferase reporter construct, *pCAGA$_{12}$-luc* was transiently transfected into those cell lines to quantitatively determine TGF-β signaling sensitivity. Stimulation with TGF-β activated *pCAGA$_{12}$-luc* activity in all 5 cell lines used (Fig. 1b). Interestingly, this increased *pCAGA$_{12}$-luc* activity was significantly reduced in EGFR over-expressing cells (A431, HN5 and 293T-EGFR), but not in the low EGFR expressing cells lines (293T and A549) when cells were treated with EGF (Fig. 1b), suggesting that EGF inhibited *pCAGA$_{12}$-luc* activity in cells with high levels of EGFR expression.

As EGF inhibited Smad3 reporter activity in cell lines expressing high EGFR levels, we next examined whether EGF had similar effects on Smad2 phosphorylation and localization. As expected, TGF-β treatments resulted in increased Smad2 phosphorylation in all 5 cell lines tested (Fig. 1c). EGF however, clearly reduced the TGF-β-mediated phospho-Smad2 levels in A431, HN5 and 293T-EGFR cells, albeit these levels were still greater than basal levels (Fig. 1c). In contrast, EGF induced no detectable difference in phospho-Smad2 levels in 293T and A549 cells (Fig. 1c). Consequently, EGF treatment caused a marked decrease of Smad2 nuclear translocalisation by TGF-β such as in A431 cells (Fig. 1d).

To confirm that EGFR activation was responsible for the observed desensitization of the TGF-β signaling, AG1478, a specific inhibitor of EGFR[38] was used. Treatment with AG1478 dramatically reduced both basal and ligand stimulated phospho-EGFR in A431, HN5

and 293T-EGFR cells (Fig. 1e). It had no effect on the TGF-β reporter activity in the normal-EGFR expressing 293T cell line (Fig. 1f). However, the EGF-mediated reduction of the TGF-β reporter activity was reversed when A431, HN5 and 293T-EGFR cells were co-treated with AG1478 (Fig. 1f), confirming that activation of over-expressed EGFR mediates the desensitization of the TGF-β signaling.

Finally, to determine whether EGFR blockade re-sensitized cells to the inhibitory effects of TGF-β, the EGFR over-expressing human head&neck tumor cells HN5 were treated with TGF-β with or without AG1478 and assessed for [^{3}H]-thymidine incorporation. Indeed, HN5 cells lost TGF-β-mediated growth arrest as it did not result in much decrease in [^{3}H]-thymidine incorporation (Fig. 1g). However, when the EGFR activity was blocked with AG1478, a reduction in [^{3}H]-thymidine incorporation greater than 50% was observed, indicating that EGFR inhibition leads to a re-sensitization of HN5 cells to TGF-β-mediated growth suppression.

EGF-Mediated Inhibition of the TGF-β pathway is not dependent on PI3-K and MEK Signaling. Two of the most documented signaling pathways activated upon EGFR phosphorylation are the Ras-MAPKs and the PI3K-Akt pathways. To examine whether these pathways were involved in the desensitization of TGF-β signaling, we used pharmacological inhibitors to block either MEK (U0126) or PI3K (LY294002) activity without effecting phospho-EGFR levels (Fig. 2a, 2b). Unlike the EGFR inhibitor AG1478, neither the MEK inhibitor U0126 nor the PI3K inhibitor LY294002 re-sensitized the TGF-β reporter activity in the EGFR over-expressing HN5 and 293T-EGFR cells (Fig. 2c, d). These results suggest that the EGF-mediated inhibition of the TGF-β pathway is not dependent on MEK and PI3K signaling.

Over-expression of EGFR mediates specifically sustained Stat3 phosphorylation and transcriptional activity. As we had previously shown that sustained Stat3 activation results in the desensitization of TGF-β signaling in the IL-6/gp130 signaling system[37], we next set out to determine whether EGF could lead to Stat3 activation. While EGF-stimulation resulted in increased phospho-EGFR, phospho-Erk1/2 and phospho-Akt levels in all 5 cell lines used (A431, HN5, 293T, 293T-EGFR and A549) without changes to total protein levels, phosphorylation of Stat3 was observed in only the EGFR over-expressing cell lines, (A431, HN5 and 293T-EGFR) (Fig. 3a). Furthermore, this EGFR over-expression-specific Stat3 activation was sustained throughout the duration of a 480 min time-course experiment (Fig. 3b). In fact the specific sustained Stat3 phosphorylation correlated with an increase in Stat3 transcriptional activation as measured by the luciferase promoter activity using the *pAPRE-luc* reporter construct in EGFR over-expression cells (Fig. 3c). There was minimal Stat3 reporter activation in the cells expressing normal or low levels of EGFR (293T and A529) (< 2-fold; data not shown). Using the EGFR inhibitor AG1478, it was further confirmed that Stat3 phosphorylation and transcriptional activity were indeed mediated by EGFR in all the cell lines examined (Fig. 3d). Finally, using the small molecular inhibitors to MEK and PI3K, the EGFR over-expression mediated Stat3 phosphorylation and transcriptional activity were determined to be independent of the MEK and PI3K pathways (Supplementary Fig. 1). Taken together, these results demonstrate that over-expression of EGFR mediates specifically sustained Stat3 phosphorylation and transcriptional activity.

EGFR mediated inhibition of the TGF-β signaling is Stat3-dependent. To verify conclusively that Stat3 mediates desensitization of the TGF-β signaling, we knocked down Stat3 protein expression by siRNA (Fig. 4a). In both A431 and HN5 cells the activity of the TGF-β reporter *pCAGA$_{12}$-luc* was increased when the endogenous Stat3 levels were knocked down (Fig. 4b), indicating that Stat3 is indeed required for the EGFR-mediated

desensitization of the TGF-β signaling. Importantly, Stat3 knock down restored TGF-β mediated growth suppression of HN5 tumor cells as determined by [^{3}H]-thymidine incorporation (Fig. 4c).

Smad7-dependent de-sensitization of TGF-β signalin by EGFR. As we have previously shown that hyper-activation of Stat3 induced expression of the negative regulator of TGF-β signaling, Smad7[37], we next examined whether the EGFR-Stat3 mediated de-sensitization of the TGF-β signaling is dependant on Smad7. Indeed, EGFR activation increased *Smad7* gene promoter activity in A431 and HN5 cells as measured by the promoter reporter *pSmad7-luc* construct (Fig. 5a). Conversely, the *pSmad7-luc* promoter activity was significantly reduced when A431 or HN5 cells were treated with the EGFR inhibitor AG1478 (Fig. 5b), demonstrating that *Smad7* promoter activity can be regulated by EGFR activation. Consequently, the Smad7 protein levels were decreased when EGFR activity is blocked by its inhibitor AG1478 (Fig. 5b). Furthermore, both the *Smad7* promoter activity and Smad7 protein expression were reduced when the Stat3 expression levels were knocked down (Fig. 5c). These data demonstrate that up-regulation of Smad7 protein levels in EGFR over-expressing tumor cells is through the Stat3 mediated *Smad7* promoter activation. To determine whether EGFR-Stat3 mediated desensitization of TGF-b signaling is due to increased Smad7 expression, we use *Smad7* siRNA to knockdown its expression (Fig. 5d). Indeed, in both A431 and HN5 tumor cells, Smad7 knockdown largely restored the TGF-β reporter activity when EGFR is activated by EGF treatment (Fig. 5e). Of note, the reporter activity in the absence of EGF treatment is also vastly increased by Smad7 knockdown, confirming the effect of the basal Smad7 expression increase in EGFR over-expressing cells, which can be reduced by EGFR inhibitor treatment (Fig. 5b). Importantly, Smad7 knockdown, like Stat3 knockdown, restored HN5 tumor cell growth inhibitory sensitivity to TGF-β (Fig. 5f).

Discussion

Ever since its discovery as one of the first receptor tyrosine kinases, EGFR and its signaling have been studied extensively. Many downstream pathways have been identified, but little is known about the difference between the normal and tumorigenic signals transduced from EGFR. It was presumed that the two main downstream pathways, Ras-MAPK and PI3K-Akt were responsible for EGFR-driven tumor growth. However, it has been difficult to detect the different mode of their activation between normal and tumor cells. At best, less than 10% elevation of MAPK activation by a constitutively active EGFR (Δ2-7EGFR)[39] was proposed to explain its tumorigenicity. Yet, these downstream signals have been employed to measure the efficacy of EGFR inhibition in cancer treatment [40-42]. The identification of Stat3 here as a molecule specifically and persistently activated by the over-expressed and tumor-associated EGFR but not by EGFR expressed at normal levels reveals a critical signaling difference by EGFR between normal and tumor cells.

In both the normal and tumor cells, MAPK (Erk1/2) and Akt activation by EGFR are rapid and transient (Fig. 3), regardless of the levels of EGFR expression. In contrast, low levels of EGFR do not activate Stat3 while only high levels of EGFR are capable of Stat3 activation. Surprisingly, Stat3 activation by high levels of EGFR is slower than the Erk1/2 or Akt activation (Fig. 3). Furthermore, the high level of EGFR-mediated Stat3 activation is persistent, unlike the transient nature of Erk1/2 and Akt activation. This is in direct contrast to cytokines such as IL-6 and IL-11 which mediated Stat3 activation rapidly and transiently[37,43]. Our previous work demonstrated in both animal models and human tissues that persistent Stat3 activation leads to stomach epithelial hyper-proliferation. It becomes clear in recent years that Stat3 is an oncogene and its activation is prevalent in many human cancers[34,44,45]. Unlike many other oncogenes, there is no genetic evidence of gain-of-function mutation. The

observation that the tumor-associated over-expressed EGFR activates Stat3 persistently identifies EGFR as an upstream molecular cause of Stat3 activation physiologically.

The tumorigenic consequence of the persistent Stat3 activation at least is partially mediated through the desensitization of TGF-β signaling via its negative regulator Smad7 expression in stomach epithelium[37]. Indeed, EGFR-mediated Stat3 activation also results in the desensitization of TGF-β signaling in many cell lines with EGFR over-expression. More importantly, in the head&neck tumor cell line with high levels of EGFR, HN5, there is a loss of cytostatic/growth inhibitory regulation by TGF-β (Fig.1), which can be reversed by blocking either EGFR or Stat3 activation (Fig. 1 and 4). Restoration of TGF-β signaling can also be achieved in those cell lines by knockdown of Smad7 expression (Fig. 5). Thus, the loss of TGF-β cytostatic regulation on normal cell growth may represent a key molecular event during many organ types of tumorigenesis driven by over-expression of EGFR through the EGFR-Stat3-Smad7-TGF-β cross-talk axis.

Loss of TGF-β sensitivity and thereby loss of cytostatic regulation may represent a key molecular event in tumor progression. Desensitization to TGF-β-mediated growth regulation can occur through the generation of loss-of-function mutations in either TGF-β receptors or downstream signaling molecules[46]. However, the overall occurrences of such mutations in human tumors are not frequent[46], suggesting that there may exist alternatives other than direct TGF-β signaling component deletion or mutation. Indeed, some early work showed that Ras inhibited TGF-β signaling through MAPK's modification of Smads[47-49] while IFN-γ inhibits TGF-β signaling through Stat1 mediated Smad7 expression[50]. We have not seen any de-sensitization of TGF-β signaling mediated by MAPKs (Erk1/2) in cells expressing either high or low levels EGFR (Fig. 3). At least in the context of stomach epithelium, Stat1 did not cause de-sensitization of TGF-β signaling[37]. Both here and our previous work[37] identify Stat3

as a key mediator of loss of TGF-β cytostatic regulation through signaling pathway cross-talk. In early tumor development where tumor growth and expansion are dominant, TGF-β acts as a tumor suppressor while it promotes invasion in late stages of tumor progression. Importantly, TGF-β signaling may be necessary for the late tumor invasion, best demonstrated in colon tumor development where deletions or mutations in the TGF-β signaling pathway gave rise to a better prognosis compared to patients with colon cancers with intact TGF-β signaling[51,52]. Desensitization of TGF-β signaling through signaling cross-talk such as EGFR-Stat3-Smad7-TGF-β demonstrated here instead of the component deletions or mutation may enable the cells to by-pass TGF-β's tumor suppressive effect in early tumor development while the pathway can be re-sensitized by different molecular means to promote tumor invasion at late stages.

Establishing the loss of cytostatic regulation by TGF-β by tumor-associated EGFR over-expression through the EGFR-Stat3-Smad7-TGF-β axis has direct implications in EGFR signaling targeted cancer therapy. Firstly, it provides some clear molecular targets for treating EGFR-driven tumors, namely, Stat3 and Smad7. Several research groups have developed therapeutics that target Stat3 with some success[36,53,54], such as administration of anti-sense oligonucleotides targeting Stat3 expressing hematological tumors in mice[53] and a synthesized triterpenoid, CDDO-Imidazolide to inhibit Stat3 phosphorylation in human myeloma and lung cancer cells[46,55]. Similar strategies may be employed to target Smad7 expression in tumors. A combination of Stat3 and/or Smad7 with EGFR targeting may provide a much more effective treatment. Secondly, to determine the efficacy of any EGFR targeted treatment, in addition to its effects on Ras-MAPK and PI3K-Akt pathways, it may be necessary to measure the effects on Stat3 activation, Smad7 activation and TGF-β signaling sensitivity. Given the availability of the sensitive luciferase reporters for Stat3, Smad7 and TGF-β signaling, it is not unreasonable to envisage them being used to directly monitor the real effects of any EGFR

targeting therapy on its more specifically tumorigenic downstream signaling. More importantly, they can be used *in vivo* in real time when coupled with live imaging techniques to optimize treatment regimen, especially regarding dosages and schedules.

Materials and Methods

Antibodies and reagents Rabbit polyclonal antibodies directed against Erk1 and Stat3 and goat polyclonal antibody directed to Akt were obtained from Santa Cruz. The phospho-Erk1/2, phospho-Akt and phospho-Stat3 rabbit polyclonal antibodies were from Cell Signaling Technology, while the mouse phospho-tyrosine monoclonal antibody (4G10) was from Upstate Biotechnology. Anti-mouse Smad2 and Actin antibodies were purchased from Transduction Laboratories and Sigma respectively. The anti-Rabbit Smad7 polyclonal antibody was obtained from Imgenex. The anti-mouse Alexa488-conjugated secondary was from Molecular Probes. The Anti-rabbit phospho-Smad2 antibody was kindly provided by Prof. P. Ten Dijke (Uppsala Branch; Ludwig Institute for Cancer Research). The mouse anti-EGF receptor antibody mAb 806 was provided by the Melbourne Centre for Clinical Sciences (Ludwig Institute for Cancer Research). The MEK inhibitor U0126 and the PI3K inhibitor LY294002 were purchased from CalBiochem. AG1478 and Recombinant mouse EGF was kindly provided by A.Prof. E. Nice (Melbourne Tumor Biology Branch; Ludwig Institute for Cancer Research) and recombinant human TGF-β1 was purchased from R & D systems. [^{3}H]-thymidine was provided by Molecular Probes. Human *Stat3* and *Smad7* siRNA were from Santa Cruz, while the fluorescein labeled control siRNA was from Qiagen.

Cells and cell culture The epidermoid carcinoma cell line A431, the head&neck carcinoma cell line HN5, the human embryonic kidney cell line HEK-293T (293T) and the EGFR-Flag tagged stably transfected HEK-293T-EGFR (293T-EGFR) cell line and the lung carcinoma cell line A549 have all been previously described[19,56-58]. The A431, HN5 and A549 cell lines were maintained in Dulbecco's Modified Eagle's Medium, while the 293T and 293T-EGFR cell lines were maintained in RPMI medium. All media contained 10% foetal bovine serum (FBS), 2 mM glutamine, 100U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated in a humidified atmosphere of 90% air and 10% CO_2 at 37°C.

Western blot analysis Cells were lysed in a lysis buffer (50mM Tris (pH 7.4), 150mM NaCl, 1% Triton-X-100, 50mM NaF, 2mM $MgCl_2$, 1mM Na_3VO_4 and protease inhibitor cocktail (Roche)) and clarified by centrifugation (13,000g for 15 min at 4°C). Proteins were then separated by SDS-PAGE (Invitrogen), blotted onto nitrocellulose and probed with the indicated primary antibodies. The signal was visualized using the ECL chemoluminescence detection kit (Amersham Biosciences) following incubation with appropriate secondary antibodies.

Luciferase assays The firefly luciferase constructs *pAPRE-luc*[59], *pSmad7-luc*[60] and *pCAGA$_{12}$-luc*[61] have been all previously described. Cells were transiently transfected with the construct using the FuGENE-6 transfection kit (Roche). After a 24 h transfection period, cells were washed with PBS and cultured with TGF-β, EGF, AG1478, U0126 and/or LY294002 at the concentrations indicated for a further 24 h. Cells were then lysed and assessed for luciferase activity using the Luciferase Reporter Assay Kit (Promega) following the manufacturer's instructions. To assess for the effects of Stat3 and Smad7 knockdown on Smad3 and Smad7 promoter activity, cells were transiently transfected with *Stat3*, *Smad7* siRNA or fluorescein-labelled control siRNA using the HiPerFect^{TM} transfection reagent (Qiagen) as per the manufacturer's instructions 24 h after transfection with *p(CAGA)$_{12}$-luc* and *pSmad7-luc* constructs.

[^{3}H]-thymidine incorporation assays Cells were plated in 96-well plates in DMEM-10% FBS and allowed to adhere overnight. Quadruplicate wells were treated with TGF-β and or AG1478 at the concentrations indicated for 48 h and then incubated with 0.2 μCi of [^{3}H]-thymidine/well for an additional 4 h. Cells were lysed with 0.5 M NaOH, harvested by using a Filtermate Harvester (Packard Instrument Co.), and the incorporated [^{3}H]-thymidine measured with a Microplate Scintillation Counter (Packard Instrument Co.). To assess for the effects of Stat3 and Smad7 knockdown on [^{3}H]-thymidine incorporation, cells were transiently

transfected with *Stat3, Smad7* siRNA or fluorescein-labelled control siRNA using the HiPerFect^{TM} transfection reagent in solution and seeded in 96-well plates 24 h prior to TGF-β treatment.

Confocal Microscopy A431 cells were seeded onto coverslips in 6-well plates in DMEM-10% FBS and allowed to adhere overnight. Cells were stimulated with or without EGF in serum-free media overnight followed by treatment with TGF-β for 15 min. Cells were then washed twice in PBS, fixed in formaldehyde and permeabilized with PBS containing 0.2% Triton-X-100. Following blocking in PBS-Tween20 containing 5% BSA, cells were stained with anti-Smad2 antibody and visualized with Alexa488-conjugated secondary antibody using confocal microscopy as described[61].

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Figure Legends

Figure 1. Over-Expression of EGFR Desensitizes the TGF-β Pathway. a. A431, HN5, 293T, 293T-EGFR and A549 cells were lysed and examined for EGFR and actin protein expression by western blot analysis as described in Materials and Methods. **b**. A431 (i), HN5 (ii), 293T (iii), 293T-EGFR (iv) and A549 (v) cells were transfected with the Smad3 reporter construct *pCAGA$_{12}$-luc* and allowed to adhere overnight. Cells were then treated with increasing concentrations of TGF-β in the presence (□) or absence (■) of EGF (20ng/ml) for a further 24 h, lysed and assessed for luciferase activity. Data are expressed as relative luciferase activity (fold change) by standardizing the luciferase activity of the un-stimulated cells to 1, and accordingly normalizing all other raw values. **c.** Cells were treated with EGF (20ng/ml) overnight in serum-free media, then stimulated with TGF-β (0, 0.2 and 2ng/ml) for 30 min as indicated above. Cells were then lysed and examined for phospho-Smad2 and total Smad2 expression by western blot analysis. **d**. A431 cells were stimulated with or without EGF (20ng/ml) overnight in serum-free media, then treated with or without TGF-β (0.2ng/ml) for 15 min as indicated above. Cells were then fixed in formaldehyde, permeablised in 0.2% Triton-X-100 and stained with anti-Smad2 antibody. Localisation of Smad2 was visualized with Alexa488-conjugated secondary antibody using confocal microscopy as described in Materials and Methods. **e.** A431, HN5 and 293T-EGFR were treated with AG1478 (0, 0.4 and 2μM) for 30 min in serum-free media, then stimulated with or without EGF (20ng/ml) for 10 min as indicated above. Cells were then lysed and examined for phospho-EGFR and total EGFR expression by western blot analysis. **f**. A431 (i), HN5 (ii), 293T (iii) and 293T-EGFR (iv) cells were transfected with *pCAGA$_{12}$-luc,* pre-treated with (■) or without (□) AG1478 for 4 h, then stimulated with TGF-β (2ng/ml) and/or EGF (20ng/ml) as indicated above for a further 24 h. Cells were then lysed and assessed for luciferase activity and expressed as outlined in Fig. 1b. **g.** HN5 cells were treated with TGF-β in the presence (■) or absence (□)

of AG1478 for 48 h. Cells were then incubated with 0.2 μCi of [^{3}H]-thymidine/well for an additional 4 h, lysed with 0.5 M NaOH, harvested, and then measured for incorporated [^{3}H]-thymidine.

Figure 2. EGF-Mediated Desensitization of the TGF-β pathway is MEK and PI3K Independent. HN5 (left) and 293T-EGFR cells (right) were treated with **a.** U0126 (0, 2 and 10μM) or **b.** LY294002 (0, 2 and 10μM) for 4 h in serum-free media, then stimulated with or without EGF (20ng/ml) for 10 min. Cells were then lysed and examined for phospho-EGFR, total EGFR, phospho-Erk1/2, total Erk1/2, phospho-Akt and total Akt expression by western blot analysis as indicated above. HN5 (left) and 293T-EGFR cells (right) were transfected with *pCAGA$_{12}$-luc,* pre-treated with (■) or without (□) **c.** U0126 or **d.** LY294002 for 4 h, then stimulated with TGF-β (2ng/ml) and/or EGF (20ng/ml) as indicated above for a further 24 h. Cells were then lysed and assessed for luciferase activity and expressed as outlined in Fig. 1b.

Figure 3. Over-expression of the EGFR Leads to Sustained Stat3 Phosphorylation and Transcriptional Activity. A431, HN5, 293T, 293T-EGFR and A549 cells were serum-starved overnight then stimulated with or without EGF (20ng/ml) for **a.** 10 min or **b.** 10, 60, 120, 240 and 480 min. Cells were then lysed and examined for phosphorylated and total expression of EGFR, Stat3, Akt and Erk1/2 as indicated above by western blot analysis as describe in Materials and Methods. **c.** A431 (i), HN5 (ii) and 293T-EGFR (iii) cells were transfected with the Stat3 reporter construct *pAPRE-luc* and allowed to adhere overnight. Cells were then treated with increasing concentrations of EGF (0 - 50ng/ml) for a further 24 h, lysed and assessed for luciferase activity and expressed as outlined in Fig. 1b. **d.** A431, HN5 and 293T-EGFR were treated with AG1478 (0, 0.4 and 2μM) for 30 min in serum-free media, then stimulated with or without EGF (20ng/ml) as indicated above. Cells were then lysed and

examined for phospho-Stat3 and total Stat3 expression by western blot analysis. Cells were also transfected with *pAPRE-luc,* pre-treated with AG1478 (0, 0.4 and 2μM) for 4 h, then stimulated with EGF (20ng/ml) as indicated above for a further 24 h. Cells were then lysed and assessed for luciferase activity and expressed as outlined in Fig. 1b.

Figure 4. EGF-Mediated Desensitization of the TGF-β Signaling Pathway is Stat3 Dependent. a. HN5 cells were transiently transfected with control or *Stat3* siRNA. After 48h, cells were lysed and examined for Stat3 and actin expression by western blot analysis as indicated above. **b**. Twenty-four h following transient transfection with *pCAGA$_{12}$-luc,* A431 (i) and HN5 (ii) cells were re-transfected with control (□) or *Stat3* (■) siRNA, seeded and allowed to adhere for 24 h. Cells were then stimulated with TGF-β (2ng/ml) and/or EGF (20ng/ml) as indicated above for a further 24 h. Cells were then lysed and assessed for luciferase activity. Data are expressed as relative luciferase activity (fold change) by standardizing the luciferase activity of the TGF-β-stimulated control siRNA transfected cells to 1, and accordingly normalizing all other raw values. **c.** Following transient transfection with control (□) or *Stat3* (■) siRNA, HN5 cells were seeded and allowed to adhere overnight. Cells were then treated with TGF-β for 48 h, incubated with 0.2 μCi of [^{3}H]-thymidine/well for an additional 4 h, lysed with 0.5 M NaOH, harvested, and then measured for incorporated [^{3}H]-thymidine.

Figure 5. EGF-Mediated Desensitization of the TGF-β Signaling Pathway is Smad7 Dependent. A431 and HN5 cells were transfected with the Smad7 reporter construct *pSmad7-luc* and allowed to adhere overnight. Cells were then treated **a.** with (■) or without (□) EGF or **b.** with (■) or without (□) AG1478 for a further 24 h, lysed and assessed for luciferase activity and expressed as outlined in Fig. 1b. **c.** Twenty-four h following transient

transfection with *pSMAD7$_{12}$-luc,* A431 and HN5 cells were re-transfected with control (□) or *Stat3* (■) siRNA, seeded and allowed to adhere for 24 h. Cells were then lysed and assessed for luciferase activity and expressed as outlined in Fig. 1b. **d.** HN5 cells were transiently transfected with control or *Smad7* siRNA. After 48h, cells were lysed and examined for Smad7 and actin expression by western blot analysis as indicated above. **e**. Twenty-four h following transient transfection with *pCAGA$_{12}$-luc,* A431 (i) and HN5 (ii) cells were re-transfected with control (□) or *Smad7* (■) siRNA, seeded and allowed to adhere for 24 h. Cells were then stimulated with TGF-β (2ng/ml) and/or EGF (20ng/ml) as indicated above for a further 24 h. Cells were then lysed and assessed for luciferase activity. Data are expressed as relative luciferase activity (fold change) as described in Fig. 4b. **f.** Following transient transfection with control (□) or *Smad7* (■) siRNA, HN5 cells were seeded and allowed to adhere overnight. Cells were then treated with TGF-β for 48 h, incubated with 0.2 μCi of [^{3}H]-thymidine/well for an additional 4 h, lysed with 0.5 M NaOH, harvested, and then measured for incorporated [^{3}H]-thymidine.

Figure 6. Schematic of EGF-Mediated Desensitization of the TGF-β Signaling Pathway. Ligand stimulation of over-expressed EGFR, leads to sustained activation of Stat3 which translocates into the nucleus and increases Smad7 protein expression. This in turn inhibits the TGF-β signaling pathway by directly competing with Smad2/3 for binding to TGF-βRI. This thereby blocks complexing of Smad2/3 with Smad4 and movement into the nucleus for specific gene transcription.

Supplementary Figure 1. EGF-mediated Stat3 activation is independent of Erk1/2 and Akt signaling. a. HN5 (left) and 293T-EGFR cells (right) were treated with U0126 (0, 2 and 10μM) for 4 h in serum-free media, then stimulated with or without EGF (20ng/ml) for 60 min. Cells were then lysed and examined for phospho-Stat3 and total Stat3 expression by western blot analysis as indicated above. Cells were also transfected with *pAPRE-luc,* pre-treated with U0126 (0, 2 and 10μM) for 4 h, then stimulated with EGF (20ng/ml) as indicated above for a further 24 h. Cells were then lysed and assessed for luciferase activity and expressed as outlined in Fig. 1b. **b.** Identical experiments as outlined above were performed using LY294002 (0, 2 and 10μM) instead of U0126.

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