

## ORIGINAL ARTICLE

Targeting Stat3 and Smad7 to restore TGF- $\beta$  cytostatic regulation of tumor cells *in vitro* and *in vivo*RB Luwor<sup>1</sup>, B Baradaran<sup>2</sup>, LE Taylor<sup>1</sup>, J Iaria<sup>1</sup>, TV Nheu<sup>1</sup>, N Amiry<sup>3</sup>, CM Hovens<sup>1,3</sup>, B Wang<sup>1</sup>, AH Kaye<sup>1</sup> and H-J Zhu<sup>1</sup>

Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) and Epidermal Growth Factor (EGF) signaling pathways are both independently implicated as key regulators in tumor formation and progression. Here, we report that the tumor-associated overexpression of epidermal growth factor receptor (EGFR) desensitizes TGF- $\beta$  signaling and its cytostatic regulation through specific and persistent Stat3 activation and Smad7 induction *in vivo*. In human tumor cell lines, reduction of TGF- $\beta$ -mediated Smad2 phosphorylation, nuclear translocation and Smad3 target gene activation were observed when EGFR was overexpressed, but not in cells that expressed EGFR at normal levels. We identified Stat3, which is activated specifically and persistently by overexpressed EGFR, as a key signaling molecule responsible for the reduced TGF- $\beta$  sensitivity. Stable knockdown of Stat3 using small hairpin RNA (shRNA) in Head and Neck (HN5) and Epidermoid (A431) tumor cell lines resulted in reduced growth compared with control shRNA-transfected cells when grown as subcutaneous tumor xenografts. Furthermore, xenografts with Stat3 knockdown displayed increased Smad3 transcriptional activity, increased Smad2 phosphorylation and decreased Smad7 expression compared with control xenografts *in vivo*. Consistently, Smad7 mRNA and protein expression was also significantly reduced when EGFR activity was blocked by a specific tyrosine kinase inhibitor, AG1478, or in Stat3 knockdown tumors. Similarly, Smad7 knockdown also resulted in enhanced Smad3 transcriptional activity *in vivo*. Importantly, there was no uptake of subcutaneous HN5 xenografts with Smad7 knockdown. Taken together, we demonstrate here that targeting Stat3 or Smad7 for knockdown results in resensitization of TGF- $\beta$ 's cytostatic regulation *in vivo*. Overall, these results establish EGFR/Stat3/Smad7/TGF- $\beta$  signaling axis driving tumor growth, which can be targeted therapeutically.

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## INTRODUCTION

Growth factor and cytokine signaling networks control many aspects of cell behavior 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- $\beta$  (TGF- $\beta$ ) and Epidermal Growth Factor (EGF) signaling pathways are both independently implicated as key regulators in tumor formation, and as such are potential therapeutic targets.<sup>1–4</sup>

TGF- $\beta$  regulates a wide range of cellular processes including cell proliferation, differentiation, migration, organization and death.<sup>3</sup> It is widely accepted that TGF- $\beta$  can act as a tumor suppressor, given the frequent occurrence of tumor formation when TGF- $\beta$  or its downstream signaling is disrupted by gene targeting in mice or the identification of loss-of-function mutations within several TGF- $\beta$  signaling components in many types of human cancers.<sup>5–7</sup> Biological responses to TGF- $\beta$  are mediated mainly by the type I (T $\beta$ RI) and II (T $\beta$ RII) transmembrane cell surface receptors,<sup>4,8</sup> which contain cytoplasmic domains with serine/threonine kinase activity. The activated ligand–receptor complex then binds and phosphorylates, through T $\beta$ RI, the intracellular signaling molecules Smad2 and Smad3.<sup>4,8,9</sup> Once phosphorylated, these regulatory Smads (R-Smad) form complexes with Smad4 (also called DPC4 for deleted in pancreatic carcinoma locus 4) and translocate into the

nucleus. In the nucleus, they associate with transcription factors to form transcriptionally active DNA complexes.<sup>4,8,9</sup>

TGF- $\beta$  signaling can be negatively regulated by multiple proteins located both extracellularly and intracellularly,<sup>4</sup> including Smad7, which directly competes with Smad2/3 for binding to T $\beta$ RI. Perturbation of TGF- $\beta$  signaling by Smad7<sup>10</sup> as a transgene leads to various forms of tumor formation in mice. However, it remains to be answered whether the TGF- $\beta$  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- $\beta$  signaling leads to tumor formation.

Epidermal growth factor receptor (EGFR) signaling is elicited by ligand binding, initiating the activation of many downstream signaling cascades including the following: Ras-Raf-Erk1/2, Phospholipase C $\gamma$ , the PTEN-regulated phosphatidylinositol 3-kinase (PI3-K)-Akt-mTOR, Src and Signal transducer and activator of transcription (Stat) family members.<sup>11</sup> In turn, these signaling intermediates trigger gene transcription of numerous proteins involved in regulating a variety of cellular functions.<sup>12</sup> Not surprisingly, genetic alterations leading to EGFR overexpression or mutation are frequently observed in almost all epithelial cancer types.<sup>13–15</sup> However, which downstream molecules are specifically activated by tumor-associated and overexpressed EGFR and are responsible for EGFR's tumorigenic function have yet to be clearly identified.

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Stat3 is a member of the Stat family of cytoplasmic transcription factors that are activated by many cytokine and growth factor receptors including the EGFR.<sup>16,17</sup> Phosphorylated Stat3 transmits its signal from the EGFR to the nucleus, where it initiates transcription of multiple genes known to promote cancer development.<sup>7,16,18</sup> Indeed, Stat3 activation has been detected at a high frequency in many types of tumors, and its activity is required for the growth of head-and-neck cancer and multiple myeloma cells.<sup>19,20</sup> We have recently discovered<sup>21</sup> that persistent activation of Stat3 causes 100% occurrence of tumor formation in a gastric mouse model. Importantly, this study identified that only persistent, not transient, activation of Stat3 desensitizes TGF- $\beta$  signaling via increased Stat3-mediated Smad7 expression, releasing cells from its cytostatic regulation, thereby allowing tumor formation.

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

## RESULTS

### Overexpression of EGFR inhibits TGF- $\beta$ signaling

Previously, we found that aberrant IL-6/gp130 signaling causes the desensitization of TGF- $\beta$  signaling in the gastric epithelium.<sup>21</sup> To test the effect of EGFR signaling on TGF- $\beta$  signaling, seven human cell lines with varying EGFR expression levels were used (Figure 1a). Of those, A431, HN5, 293T-EGFR and U87MG-EGFR overexpress EGFR, whereas the 293T, A549 and U87MG do not. The Smad3 luciferase reporter construct *pCAGA<sub>12</sub>-luc* was transiently transfected into those cell lines to quantitatively determine TGF- $\beta$  signaling sensitivity. Stimulation with TGF- $\beta$ -activated *pCAGA<sub>12</sub>-luc* activity was seen in all seven cell lines used (Figure 1b). Interestingly, this increased *pCAGA<sub>12</sub>-luc* activity was significantly reduced in all four EGFR-overexpressing cells, but not in the low levels of EGFR-expressing cell lines when cells were cotreated with EGF (Figure 1b), suggesting that EGF inhibited *pCAGA<sub>12</sub>-luc* activity in cells with high levels of EGFR expression. Furthermore, EGF reduced the TGF- $\beta$ -mediated phospho-Smad2 levels in the EGFR-overexpressing cell lines A431, HN5 (Figure 1c) and 293T-EGFR cells (Supplementary Figure 1A), without affecting phospho-Smad2 levels in normal EGFR-expressing cells (293T and A549; Supplementary Figure 1A). Consequently, EGF treatment caused a marked decrease of Smad2 nuclear localization by TGF- $\beta$  in A431 cells (Figure 1d). In addition, EGF reduced the expression of TGF- $\beta$ -induced p21WAF1, an inhibitor of cyclin-dependent kinases and mediator of growth arrest (Supplementary Figure 1B).

To confirm that EGFR activation was responsible for the observed desensitization of the TGF- $\beta$  signaling, AG1478, a specific inhibitor of EGFR<sup>22</sup> (Supplementary Figure 1C), was used. EGF-mediated reduction of the TGF- $\beta$  reporter activity was reversed when A431 and HN5 cells were cotreated with AG1478 (Figure 1e), confirming that activation of overexpressed EGFR mediates the desensitization of the TGF- $\beta$  signaling. Furthermore, blockade of EGFR activity in HN5 cells by AG1478 resensitized these cells to the growth-inhibitory effects of TGF- $\beta$ , producing a reduction in [<sup>3</sup>H]-thymidine incorporation >50% (Figure 1f).

### EGF-mediated inhibition of TGF- $\beta$ signaling is not dependent on PI3-K and MEK activity

The two most documented signaling pathways activated upon EGFR phosphorylation are the Ras-MAPKs (MEKs) and the PI3-K/Akt pathways. Both pathways have been implicated in modulating Smad activation.<sup>23–25</sup> To examine whether these pathways were involved in the desensitization of TGF- $\beta$  signaling by

overexpressed EGFR, we used pharmacological inhibitors to block either MEK (U0126) or PI3-K (LY294002) activity without affecting phospho-EGFR levels (Figures 2a and b; Supplementary Figures 2A and B). Unlike the EGFR inhibitor AG1478, neither U0126 nor LY294002 resensitized the TGF- $\beta$  reporter activity in HN5 and 293T-EGFR cells (Figure 2c and Supplementary Figure 2C), suggesting that the inhibition of the TGF- $\beta$  pathway mediated by the overexpression of EGFR is not dependent on MEK and PI3K signaling.

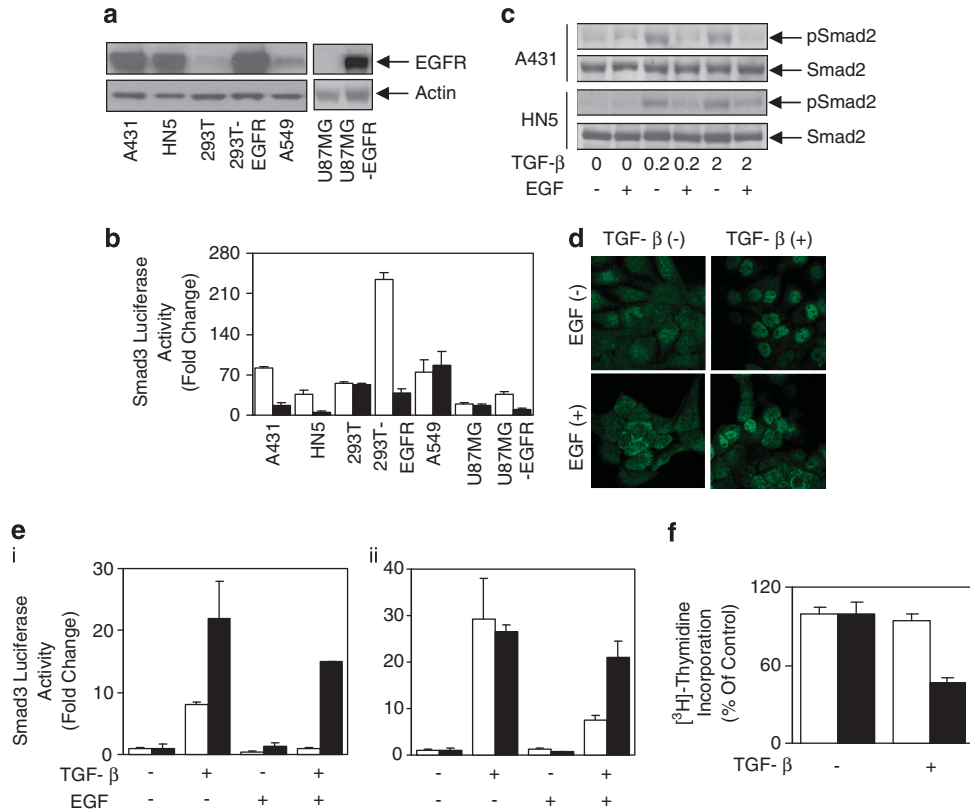
### EGFR overexpression induces specifically sustained Stat3 phosphorylation and transcriptional activity

As we had previously shown that sustained Stat3 activation results in the desensitization of TGF- $\beta$  signaling in the IL-6/gp130 signaling system,<sup>21</sup> we next set out to determine whether EGFR activated Stat3 in several human cell lines. Although EGF stimulation resulted in increased phospho-EGFR, phospho-Erk1/2 and phospho-Akt levels in all five cell lines used, phosphorylation of Stat3 was only observed in cell lines overexpressing EGFR (A431, HN5 and 293T-EGFR) (Figure 3a). Furthermore, this EGFR-overexpression-specific Stat3 activation was sustained for at least 8 h post EGF stimulation (Figure 3b). In fact, the EGFR-mediated Stat3 phosphorylation observed correlated with an increase in Stat3 transcriptional activation as measured by the luciferase activity using the *pAPRE-luc* reporter construct in EGFR-overexpressing cells (Supplementary Figures 3A and B). TGF- $\beta$  stimulation did not affect EGF-mediated Stat3 activity (Supplementary Figure 3C). There was minimal Stat3 reporter activation (<twofold) in the cells expressing normal or low levels of EGFR (293T, A549 and U87MG; Supplementary Figure 3B). Taken together, these results demonstrate that overexpression of EGFR mediates specifically sustained Stat3 phosphorylation and transcriptional activity.

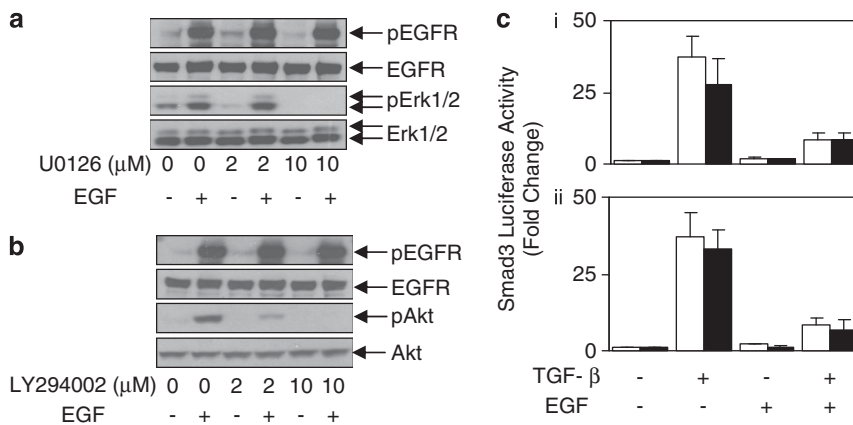
Our results so far demonstrate an inverse correlation between the specific activation of Stat3 and the TGF- $\beta$  signaling sensitivity specifically in cells overexpressing EGFR. To verify conclusively that Stat3 mediates desensitization of the TGF- $\beta$  signaling, we knocked down Stat3 protein expression by small interfering RNA (siRNA) (Supplementary Figure 4A). The activity of the TGF- $\beta$  reporter *pCAGA<sub>12</sub>-luc* was increased when the endogenous Stat3 levels were knocked down in both A431 and HN5 cells (Figure 3c), indicating that Stat3 is indeed required for the overexpressed EGFR-mediated desensitization of the TGF- $\beta$  signaling. Importantly, Stat3 knockdown restored TGF- $\beta$ -mediated growth suppression of HN5 cells (Figure 3d).

### Stable knockdown of Stat3 in HN5 and A431 cells results in reduced tumor growth

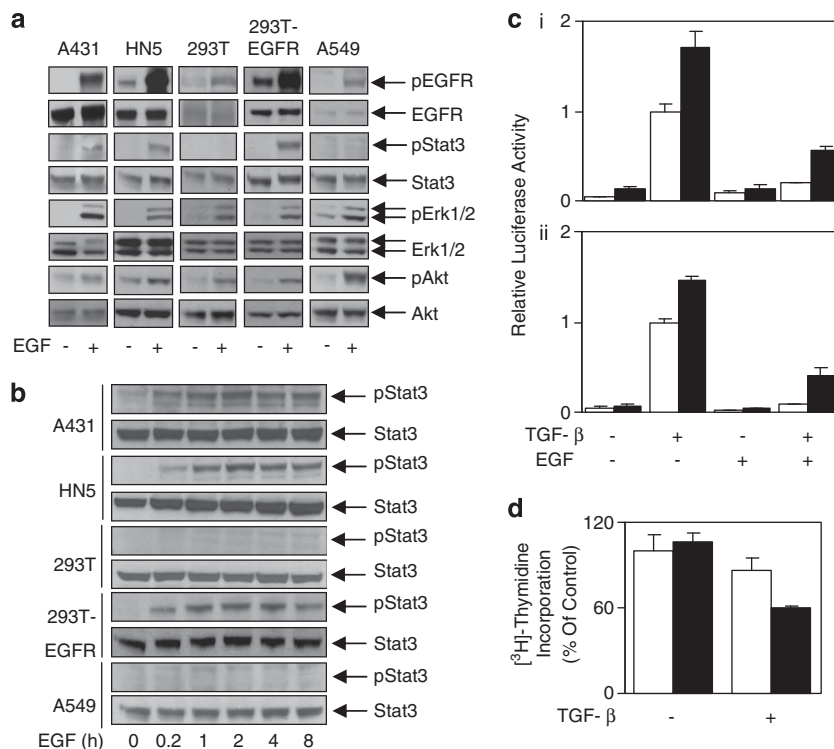
To confirm our *in vitro* results and to examine the role of Stat3 in an *in vivo* setting, we generated HN5 and A431 cells with stable knockdown of Stat3 expression using Stat3-specific small hairpin RNA (shRNA). HN5 and A431 cells with reduced Stat3 expression (designated S1, S2 and S3 for HN5 and A-S1 and A-S2 for A431 cells) expressed similar levels of EGFR compared with control cells (Figure 4a and Supplementary Figure 4B). As expected, S1, S2 and S3 cells also displayed reduced EGF-mediated Stat3 transcriptional activity (Supplementary Figure 4C) and phosphorylated Stat3 compared with the control HN5 cell line, although no effect was seen on phosphorylated EGFR or phospho-Erk1/2 levels (Supplementary Figure 4D). Most importantly, the tumor growth of these clones was significantly reduced when grown as xenografts in BALB/c<sup>nu/nu</sup> mice, indicating a key role of Stat3 in mediating tumorigenicity of HN5 and A431 cells (Figure 4b and Supplementary Figure 4E). Two tumors from each HN5 subclone were removed with subsequent western blot analysis of these tumors, showing that the knockdown of Stat3 had remained intact *in vivo* throughout the course of the experiment (Supplementary



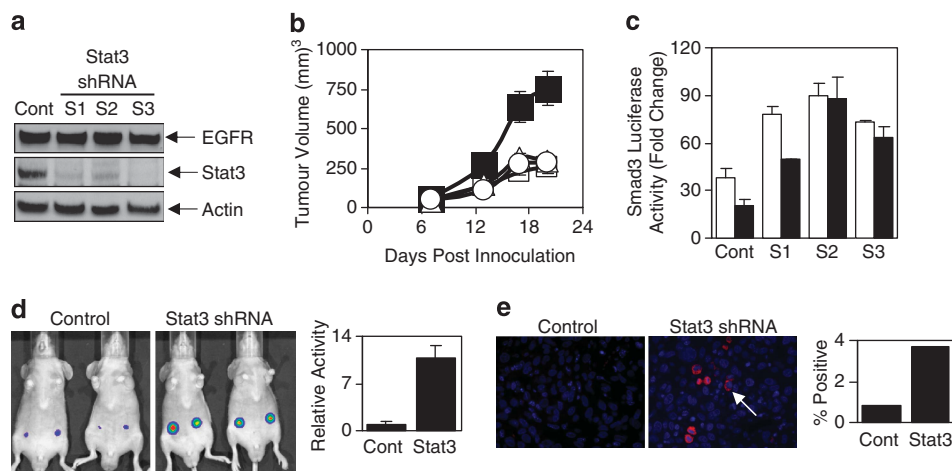
**Figure 1.** Overexpression of EGFR desensitizes the TGF- $\beta$  pathway. **(a)** Cell lines as indicated were lysed and examined for EGFR and Actin protein expression or **(b)** transfected with the Smad3 reporter construct *pCAGA<sub>12</sub>-luc* and allowed to adhere overnight. Cells were then treated with 2 ng/ml of TGF- $\beta$  in the presence ( $\square$ ) or absence ( $\blacksquare$ ) of EGF (20 ng/ml) for a further 24 h, and then lysed and assessed for luciferase activity. Data are expressed as relative Smad3 luciferase activity (fold change) by standardizing the luciferase activity of unstimulated cells to 1, and accordingly normalizing all other raw values. **(c)** Cells were treated with EGF (20 ng/ml) overnight, then stimulated with TGF- $\beta$  (0, 0.2 and 2 ng/ml) for 30 min and lysed and examined for phospho-Smad2 and total Smad2 expression. **(d)** A431 cells were stimulated with or without EGF (20 ng/ml) overnight, then treated with or without TGF- $\beta$  (0.2 ng/ml) for 15 min. Cells were then fixed in formaldehyde, permeabilized in 0.2% Triton-X-100 and stained with anti-Smad2 antibody. Localization of Smad2 was visualized with Alexa488-conjugated secondary antibody using confocal microscopy. **(e)** A431 (i) and HN5 (ii) cells were transfected with *pCAGA<sub>12</sub>-luc*, pretreated with ( $\blacksquare$ ) or without ( $\square$ ) AG1478 for 4 h, and then stimulated with TGF- $\beta$  (2 ng/ml) and/or EGF (20 ng/ml) for a further 24 h. Cells were then lysed and assessed for luciferase activity and expressed as outlined in Figure 1b. **(f)** HN5 cells were treated with or without TGF- $\beta$  in the presence ( $\blacksquare$ ) or absence ( $\square$ ) of AG1478 for 48 h. Cells were then incubated with 0.2  $\mu$ Ci of [ $^3$ H]-thymidine/well for 4 h, lysed with 0.5 M NaOH, harvested and then measured for incorporated [ $^3$ H]-thymidine.



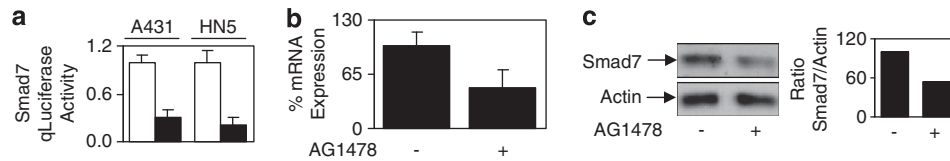
**Figure 2.** EGF-mediated desensitization of the TGF- $\beta$  pathway is MEK and PI3K independent. HN5 cells were treated with **(a)** U0126 (0, 2 and 10  $\mu$ M) or **(b)** LY294002 (0, 2 and 10  $\mu$ M) for 4 h, then stimulate with or without EGF (20 ng/ml) for 10 min. Cells were then lysed and examined for phosphorylated and total expression of EGFR, Akt and Erk1/2. **(c)** HN5 cells were transfected with *pCAGA<sub>12</sub>-luc*, pre-treated with ( $\blacksquare$ ) or without ( $\square$ ) (i). U0126 and (ii). LY294002 for 4 h, then stimulated with TGF- $\beta$  (2 ng/ml) and/or EGF (20 ng/ml) for a further 24 h. Cells were then lysed and assessed for luciferase activity and expressed as outlined in Figure 1b.



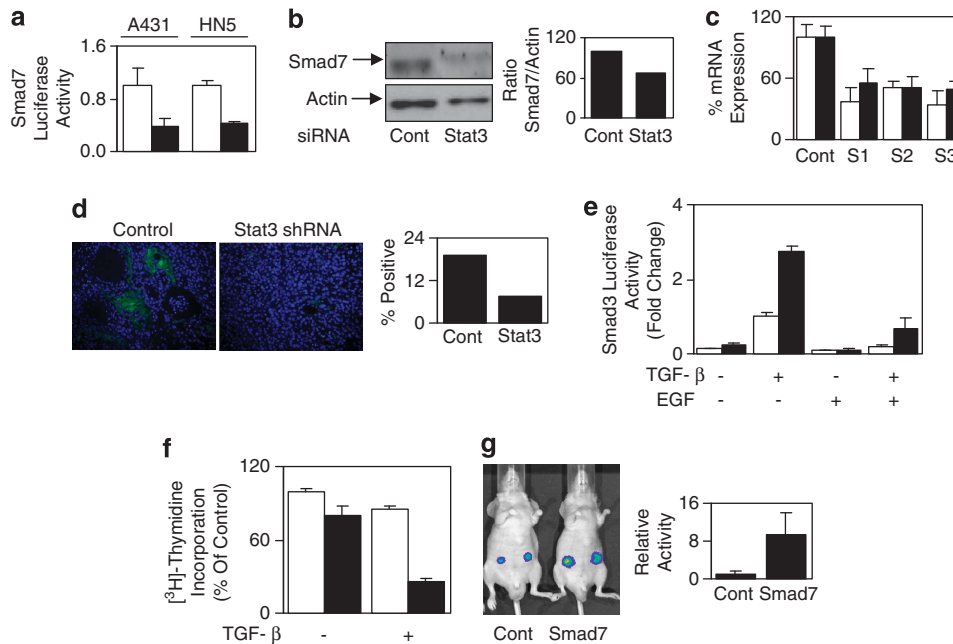
**Figure 3.** Overexpression of the EGFR leads to sustained Stat3 phosphorylation and desensitized TGF- $\beta$  signaling. Cells were stimulated with or without EGF (20 ng/ml) for (a) 10 min or (b) 0.2, 1, 2, 4 and 8 h and then lysed and examined for phosphorylated and total expression of EGFR, Stat3, Akt and Erk1/2. (c) At 24 h following transient transfection with *pCAGA<sub>12</sub>-luc*, A431 (i) and HN5 (ii) cells were retransfected with control (□) or Stat3 (■) siRNA, seeded and allowed to adhere for 24 h. Cells were then stimulated with TGF- $\beta$  (2 ng/ml)  $\pm$  EGF (20 ng/ml) as indicated above for a further 24 h. Cells were then lysed and assessed for luciferase activity and expressed as relative luciferase activity (fold change). (d) Following transient transfection with control (□) or Stat3 (■) siRNA, HN5 cells were seeded and allowed to adhere overnight. Cells were then treated with or without TGF- $\beta$  for 48 h and measured for incorporated [<sup>3</sup>H]-thymidine as outlined in Figure 1f.



**Figure 4.** Stable Stat3 knockdown desensitizes the TGF- $\beta$  signaling pathway *in vitro* and *in vivo*. (a) HN5-control and Stat3 shRNA clones (S1, S2, S3) were lysed and examined for EGFR, Stat3 and Actin protein expression or (b) inoculated subcutaneously into both flanks of BALB/c<sup>nu-/-nu-/-</sup> female mice and measured for tumor volume ( $n = 10$ /group) as indicated in Materials and methods; HN5-control (■) or Stat3 shRNA (open symbols). (c) HN5-control, S1, S2 and S3 cells were transfected with *pCAGA<sub>12</sub>-luc* and allowed to adhere overnight. Cells were then treated with 2 ng/ml of TGF- $\beta$  in the presence (□) or absence (■) of EGF (20 ng/ml) for a further 24 h, and then either lysed and assessed for luciferase activity as outlined in Figure 1b or (d) inoculated subcutaneously into both flanks of BALB/c<sup>nu-/-nu-/-</sup> female mice and analyzed for Smad3 reporter activity using the IVIS lumina Animal Imager as outlined in Materials and methods 24 h post inoculation. Cells were infected with the adenoviral smad3 reporter construct (*Ad-pCAGA-luc*) overnight, 24 h before inoculation into mice. Analysis of total photon emissions (Smad3 transcriptional activity) was performed using the IVIS lumina image analysis software and was presented relative to control tumors  $\pm$  s.d. ( $n = 4$ ). (e) HN5-control and Stat3 shRNA xenografts were removed and fixed in formaldehyde. Sections were stained for phospho-Smad2 expression and visualized with Alexa546-conjugated secondary antibody using confocal microscopy. Percentage positive staining was determined by counting approximately 3000 cells per group.



**Figure 5.** EGFR-mediated desensitization of the TGF- $\beta$  signaling pathway is Smad7 dependent. (a) A431 and HN5 cells were transfected with the Smad7 reporter construct *pSmad7-luc* and allowed to adhere overnight. Cells were then treated with (■) or without (□) AG1478 for a further 24 h, lysed and assessed for luciferase activity and expressed as outlined in Figure 1b. HN5 cells were treated with or without AG1478 overnight and then analyzed for Smad7 (b) mRNA and (c) protein expression.



**Figure 6.** Stat3 knockdown desensitizes the TGF- $\beta$  signaling pathway by reducing Smad7 expression. (a) At 24 h following transient transfection with *pSMAD7<sub>12</sub>-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 Figure 1b. (b) HN5 cells were transiently transfected with control or *Smad7* siRNA. After 48 h, cells were lysed and examined for Smad7 and Actin expression. (c) HN5-control or *Stat3* shRNA clones (S1, S2, S3) were lysed and analyzed for *Stat3* (□) and *Smad7* (■) mRNA expression. (d) HN5-control and *Stat3* shRNA xenografts from Figure 4e were stained for Smad7 expression and visualized with Alexa488-conjugated secondary antibody using confocal microscopy. Percentage positive staining was determined by counting approximately 4000 cells per group. (e) At 24 h following transient transfection with *pCAGA<sub>12</sub>-luc*, HN5 cells were re-transfected with control (□) or *Smad7* (■) siRNA, seeded and allowed to adhere for 24 h. Cells were then stimulated with TGF- $\beta$  (2 ng/ml)  $\pm$  EGF (20 ng/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 Figure 3c. (f) Following transient transfection with control (□) or *Smad7* (■) siRNA, HN5 cells were seeded and allowed to adhere overnight. Cells were then measured for incorporated [<sup>3</sup>H]-thymidine as outlined in Figure 1f. (g) HN5-control or *Smad7* shRNA cells were infected with the adenoviral *smad3* reporter construct (*Ad-pCAGA-luc*) and then inoculated subcutaneously into both flanks of BALB/c<sup>nu-/nu-</sup> female mice. *Smad3* reporter activity was analyzed as outlined in Figure 4d ( $n = 4$ ).

Figure 4F). As with transient knockdown of *Stat3*, stable knockdown of *Stat3* increased the TGF- $\beta$ -mediated activity of *pCAGA<sub>12</sub>-luc* and circumvented the inhibitory effects of EGF stimulation (Figure 4c). Furthermore, *pCAGA<sub>12</sub>-luc* activity was also increased by approximately 10-fold in HN5 cells with stable *Stat3* knockdown compared with control cells *in vivo* (Figure 4d), whereas phospho-Smad2 expression was detected in approximately 4% of HN5 cells with *Stat3* knockdown versus 1% of HN5 control cells *in vivo* (Figure 4e). These data confirm that *Stat3* is required for the overexpressed EGFR-mediated desensitization of the TGF- $\beta$  signaling.

#### Smad7-dependent desensitization of TGF- $\beta$ signaling by EGFR

As IL-6/gp130 signaling sustained *Stat3* activation and induced the expression of the negative regulator of TGF- $\beta$  signaling,

*Smad7*,<sup>21</sup> we next examined whether the EGFR-*Stat3*-mediated desensitization of TGF- $\beta$  signaling is also dependent on *Smad7*. Indeed, blockade of EGFR activation by AG1478 significantly reduced *Smad7* gene promoter activity in A431 and HN5 cells (Figure 5a), whereas EGF stimulation enhanced *Smad7* promoter activity (Supplementary Figure 5A). AG1478 treatment also reduced *Smad7* mRNA and protein levels in HN5 cells (Figures 5b and c). In addition, EGF inhibited TGF- $\beta$ -mediated TGF $\beta$ RI/Smad2 association presumably through *Smad7*'s well-recognized role of competing for TGF $\beta$ RI binding (Supplementary Figure 5B). Furthermore, both *Smad7* promoter activity and *Smad7* protein expression were reduced when the *Stat3* expression levels were knocked down transiently by siRNA (Figure 6a and b). Comparison of HN5 control and stable *Stat3* knockdown xenografts also revealed reduced *Smad7* mRNA expression (Figure 6c) and protein expression *in vivo* (Figure 6d). These

data demonstrate that upregulation of Smad7 protein levels in EGFR-overexpressing tumor cells is through Stat3-mediated *Smad7* promoter activation. To determine whether EGFR-Stat3-mediated desensitization of TGF- $\beta$  signaling is due to increased Smad7 expression, we use *Smad7* siRNA to knock down its expression. Indeed, in HN5 tumor cells, Smad7 knockdown largely restored the TGF- $\beta$  reporter activity when EGFR is activated by EGF treatment (Figure 6e). It is noteworthy that the reporter activity in the absence of EGF treatment is also vastly increased by Smad7 knockdown, confirming the effect of increased basal Smad7 expression in EGFR-overexpressing cells. Importantly, Smad7 knockdown, similar to Stat3 knockdown, restored HN5 tumor cell growth inhibitory sensitivity to TGF- $\beta$  (Figure 6f). Finally, and similar to stable knockdown of Stat3, stable Smad7 knockdown by shRNA in HN5 cells resulted in increased *pCAGA*<sub>12</sub>-luc activity by approximately 10-fold compared with control cells *in vivo* (Figure 6g). Taken together, this signaling pathway cross talk mediated by EGFR–Stat3–Smad7–TGF- $\beta$  operates specifically in tumor-associated EGFR-overexpressing cells.

## 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 PI3-K/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, <10% elevation in MAPK activation by a constitutively active EGFR ( $\Delta$ 2-EGFR)<sup>26</sup> was proposed to explain its tumorigenicity. Yet these downstream signals have been used to measure the efficacy of EGFR inhibition in cancer treatment.<sup>27–29</sup> The identification of Stat3 here as a molecule specifically and persistently activated by the overexpressed 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, regardless of the levels of EGFR expression. In contrast, low levels of EGFR do not activate Stat3, whereas only high levels of EGFR are capable of Stat3 activation. It appears that a threshold of approximately 10<sup>6</sup> EGFR/cell is required for EGF-mediated Stat3 activity based on the cell lines tested here. Surprisingly, Stat3 activation by high levels of EGFR is slower than the Erk1/2 or Akt activation (Figure 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.<sup>21,30</sup> Our previous work demonstrated, in both animal models and human tissues, that persistent Stat3 activation leads to stomach epithelial hyperproliferation. It has become clear that Stat3 is an oncogene, and its activation is prevalent in many human cancers;<sup>16,31,32</sup> however, unlike many other oncogenes, there is no genetic evidence of gain-of-function mutation. Our observation that the tumor-associated overexpressed EGFR activates Stat3 persistently identifies EGFR as a physiologically relevant upstream molecular cause of Stat3 activation.

The tumorigenic consequence of the persistent Stat3 activation is at least partially mediated through the desensitization of TGF- $\beta$  signaling via its negative regulator Smad7 in stomach epithelium.<sup>21</sup> Indeed, EGFR-mediated Stat3 activation also results in the desensitization of TGF- $\beta$  signaling in many tumor cell lines with EGFR overexpression. More importantly, in the head-and-neck

tumor cell line with high levels of EGFR, HN5, there is a loss of cytostatic/growth-inhibitory regulation by TGF- $\beta$  (Figure 1), which can be reversed by blocking either EGFR activation or reducing Stat3 or Smad7 expression (Figures 1, 3 and 6). Restoration of TGF- $\beta$  signaling as evident by enhanced cytosolic and nuclear phospho-Smad2 can also be achieved by stable knockdown of Stat3 or Smad7 expression *in vivo* (Figures 4 and 6).<sup>33</sup> Thus, the loss of TGF- $\beta$  cytostatic regulation on normal cell growth may represent a key molecular event during tumorigenesis driven by overexpression of EGFR through the EGFR–Stat3–Smad7–TGF- $\beta$  signaling axis.

Loss of TGF- $\beta$  sensitivity and thereby loss of cytostatic regulation may represent a key molecular event in tumor progression. Desensitization to TGF- $\beta$ -mediated growth regulation can occur through the generation of loss-of-function mutations in either TGF- $\beta$  receptors or downstream signaling molecules.<sup>34</sup> However, the overall occurrences of such mutations in human tumors are not frequent,<sup>34</sup> suggesting that there may exist alternatives other than direct deletion or mutation of TGF- $\beta$  signaling components. Indeed, some early work showed that Ras inhibited TGF- $\beta$  signaling through MAPK's modification of Smads,<sup>24,27,35</sup> whereas IFN- $\gamma$  inhibits TGF- $\beta$  signaling through Stat1-mediated Smad7 expression.<sup>36</sup> We have not seen any desensitization of TGF- $\beta$  signaling mediated by MAPKs (Erk1/2) in cells expressing high levels of EGFR (Figure 2). At least in the context of the stomach epithelium, Stat1 did not cause desensitization of TGF- $\beta$  signaling.<sup>21</sup> We identify Stat3 as a key mediator of loss of TGF- $\beta$  cytostatic regulation through signaling pathway cross talk *in vivo* both here and previously.<sup>21</sup> In early tumor development where tumor growth and expansion are dominant, TGF- $\beta$  acts as a tumor suppressor, whereas it promotes invasion in late stages of tumor progression. Importantly, TGF- $\beta$  signaling may be necessary for the late tumor invasion, best demonstrated in colon tumor development where deletions or mutations in the TGF- $\beta$  signaling pathway gave rise to a better prognosis compared with patients with colon cancers with intact TGF- $\beta$  signaling.<sup>37,38</sup> Desensitization of TGF- $\beta$  signaling through cross talk such as EGFR–Stat3–Smad7–TGF- $\beta$  demonstrated here, instead of the deletion or mutation of TGF- $\beta$  signaling, may enable the cells to bypass TGF- $\beta$ 's tumor-suppressive effect in early tumor development, although the pathway can be resensitized by different molecular means to promote tumor invasion at late stages.

Establishing the loss of cytostatic regulation by TGF- $\beta$  by tumor-associated EGFR overexpression through the EGFR–Stat3–Smad7–TGF- $\beta$  axis has direct implications in EGFR signaling-targeted cancer therapy. First, 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,<sup>20,39,40</sup> such as administration of antisense oligonucleotides targeting Stat3-expressing hematological tumors in mice<sup>39</sup> and a synthesized triterpenoid, CDDO-lmidazolide, to inhibit Stat3 phosphorylation in human myeloma and lung cancer cells.<sup>34,41</sup> A similar strategy may be used to target Smad7 expression in tumors. A combination of Stat3 and/or Smad7 with EGFR targeting may provide a much more effective treatment. Second, to determine the efficacy of any EGFR-targeted treatment, in addition to its effects on Ras/MAPK and PI3-K/Akt pathways, it may be necessary to measure the effects on Stat3 activation, Smad7 expression and TGF- $\beta$ -signaling sensitivity. Given the availability of the sensitive luciferase reporters for Stat3, Smad7 and TGF- $\beta$  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, TGF $\beta$ RI, p21WAF1 and Stat3 and the polyclonal goat Akt antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The phospho-Erk1/2, phospho-Stat3 and Phospho-Akt rabbit polyclonal antibodies were from Cell Signaling Technology (Danvers, MA, USA), whereas the mouse phospho-tyrosine monoclonal antibody (4G10) was from Upstate Biotechnology (Lake Placid, NY, USA). The anti-mouse Actin and Smad2 antibodies were purchased from Sigma (St Louis, MO, USA) and BD transduction Laboratories (Rockville, MD, USA), respectively. The Anti-rabbit phospho-Smad2 antibody was kindly provided by Professor P Ten Dijke (Leiden University Medical Center, The Netherlands). The anti-rabbit Smad7 antibody was from Imgenex (Imgenex Corporation, San Diego, CA, USA). The anti-rabbit Alexa<sup>488</sup>-conjugated and Alexa<sup>546</sup>-conjugated secondary antibodies and the [<sup>3</sup>H]-thymidine were from Invitrogen (Mulgrave, VIC, Australia). The mouse anti-EGF receptor antibody was provided by the Melbourne Centre for Clinical Sciences (Ludwig Institute for Cancer Research). AG1478 and Recombinant mouse EGF were kindly provided by the Melbourne Tumor Biology Branch, Ludwig Institute for Cancer Research, and recombinant human TGF- $\beta$ 1 was purchased from R & D Systems (Minneapolis, MN, USA). The MEK inhibitor U0126 and the PI3K inhibitor LY294002 were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Human *Stat3* and *Smad7* siRNAs were from Santa Cruz, whereas the fluorescein-labeled control siRNA was from Qiagen (Qiagen, SA Biosciences, Frederick, MD, USA).

### Cells and cell culture

The epidermoid carcinoma cell line A431, the head-and-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, the lung carcinoma cell line A549, the glioblastoma cell line U87MG and EGFR stable transfected U87MG-EGFR have all been previously described.<sup>42–46</sup> The HN5-Stat3 shRNA clones (designated S1, S2 and S3) and A431-Stat3 shRNA clones (designated A-S1 and A-S2) were generated by transfecting cells with the SureSilencing *Stat3-shRNA* construct (Qiagen) using FuGENE HD transfection reagent (Roche, Basel, Switzerland) following the manufacturer's instructions, and selected for using Geneticin (Roche). Similarly, the HN5-Smad7 shRNA clones were generated by transfecting cells with the SureSilencing *Smad7-shRNA* construct (Qiagen) as above. Control HN5 cells were generated by stably transfecting a negative-control shRNA (Qiagen). All cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS) (DKSH, Hallam, VIC, Australia), 2 mM glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Invitrogen). Cells were incubated in a humidified atmosphere of 90% air and 10% CO<sub>2</sub> at 37 °C.

### Western blot and immunoprecipitation analysis

Cells were lysed in a lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton-X-100, 50 mM NaF, 2 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor cocktail (Roche)) and clarified by centrifugation (13 000g for 15 min at 4 °C). Tumor xenografts were snap-frozen and lysed in RIPA buffer (50 mM Tris (pH 7.4), 5 mM EDTA, 150 mM NaCl, 0.5% Na Deoxycholate, 0.5% SDS, 10 mM NaF and protease inhibitor cocktail (Roche)) and also clarified by centrifugation. For Immunoprecipitation experiments, anti-TGF $\beta$ RI antibody and protein-A-Sepharose beads (Amersham Biosciences Corporation, Piscataway, NJ, USA) were added to clarified proteins for 4 h at 4 °C. Immune complexes were washed three times in ice-cold PBS. 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 (GE Healthcare, Rydellmere, NSW, Australia) following incubation with appropriate secondary antibodies.

### Luciferase assays

The firefly luciferase constructs *pAPRE-luc*,<sup>47</sup> *pSmad7-luc*<sup>48</sup> and *pCAGA<sub>12</sub>-luc*<sup>49</sup> have all been previously described. Cells were transiently transfected with the construct using the FuGENE HD transfection kit (Roche). After a 24-h transfection period, cells were washed with PBS and cultured with TGF- $\beta$ , 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 Corporation, Madison, WI, USA) 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* or fluorescein-labeled control siRNA using the HiPerFect transfection reagent (Qiagen) as per the manufacturer's instructions 24 h after transfection with *p(CAGA)<sub>12</sub>-luc* and *pSmad7-luc* constructs. The creation of the Adenoviral Smad3 reporter (*Ad-pCAGA-luc*) has been described previously.<sup>50</sup>

### [<sup>3</sup>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- $\beta$  and/or AG1478 at the concentrations indicated for 48 h and then incubated with 0.2  $\mu$ Ci of [<sup>3</sup>H]-thymidine/well for an additional 4 h. Cells were lysed with 0.5 M NaOH, harvested using a Filtermate Harvester (Packard Instrument Co., Meriden, CT, USA) and the incorporated [<sup>3</sup>H]-thymidine measured with a Microplate Scintillation Counter (Packard Instrument Co.). To assess for the effects of Stat3 and Smad7 knockdown on [<sup>3</sup>H]-thymidine incorporation, cells were transiently transfected with *Stat3*, *Smad7* siRNA or fluorescein-labeled control siRNA using the HiPerFect transfection reagent in solution and seeded in 96-well plates 24 h before TGF- $\beta$  treatment.

### Immunofluorescence staining and confocal microscopy

A431 cells were seeded onto coverslips in six-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- $\beta$  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. Tumor xenografts were excised from mice at the end of the experiment and processed in 10% formalin/PBS before being embedded in paraffin. Tumor sections (4  $\mu$ m) were then dewaxed, fixed, permeabilized and blocked as described above and stained with either pSmad2 or Smad7 antibody. Visualization was achieved with either Alexa<sup>546</sup> or Alexa<sup>488</sup>-conjugated secondary antibody using confocal microscopy as described.<sup>49</sup>

### Tumor growth and bioluminescence imaging *in vivo*

A431 cells and all derivatives ( $3 \times 10^6$ ) or HN5 cell and all derivatives ( $1 \times 10^7$ ) were inoculated subcutaneously into both flanks of 4- to 6-week-old BALB/c<sup>nu/nu</sup> female mice (Animal Research Center, WA, Australia). Tumor volume in mm<sup>3</sup> was determined using the formula (length  $\times$  width<sup>2</sup>)/2, where length was the longest axis and width the measurement at right angles to the length.<sup>51</sup> This research project was approved by the Animal Ethics Committee of the Ludwig Institute for Cancer Research and Department of Surgery, University of Melbourne at the Royal Melbourne Hospital. For bioluminescence imaging, cells were infected with an Adenoviral Smad3 reporter construct (*Ad-pCAGA<sub>12</sub>-Luc*) 24 h before subcutaneous inoculation into BALB/c<sup>nu/nu</sup> female mice. Mice were then injected intraperitoneally with 150 mg/kg D-luciferin (Xenogen Corp., Alameda, CA, USA) in PBS, anesthetized with isoflurane and placed under the IVIS camera (Xenogen Corp). The bioluminescence images were recorded between 10 and 20 min after each D-luciferin injection, and bioluminescence intensity was quantified as the sum of detected photons per second within the region of interest using the LivingImage software (Xenogen Corp).

### RNA extraction and qRT-PCR

RNA from cells or xenograft lysates were extracted using TRIzol reagent (Invitrogen), and converted to cDNA using SuperScript III First-Strand Synthesis SuperMix as per the manufacturer's instructions (Invitrogen). RT-PCR was performed using an ABI 7700 Real-Time PCR system (Applied Biosystems, Life Technologies, Mulgrave, Victoria, Australia). The following primers were used: *Stat3*, 5'-GAGGACTGAGCATCGAGCA-3' (forward) and 5'-CATGTGATCTGACACCCTGAA-3' (reverse); *Smad7*, 5'-CTGCAACCCCATCACCTTA-3' (forward) and 5'-CCCTGTTTCAGCGGAGGAA-3' (reverse); and *GAPDH*, 5'-TGCACCACCAACTGCTTAGC-3' (forward) and 5'-GGCATGGAC TGTGGTCATGAG-3' (reverse). Triplicate reactions with SYBR Green (Invitrogen) were performed using a two-step amplification program of initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 30 s. A melting-curve analysis step was carried out at the end of the amplification, consisting of denaturation at 95 °C for 1 min and re-annealing at 55 °C for 1 min. Each change in gene expression is expressed as percentage compared with the control and normalized to *GAPDH* Data acquisition, and analysis was performed using the ABI 7700 Real-Time PCR system software.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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