Author's response to reviews

Title: High-level inducible Smad4-reexpression is associated with gene expression profiles that predict a preferential role of Smad4 in extracellular matrix composition

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Author's response to reviews: see over
Dear editorial team,

We would like to thank the reviewers for the comments and constructive suggestions.

**point-by-point reply**

Referee Friedrich

We followed his helpful suggestions for formal improvement of the manuscript:

- The Introduction was shortened by one third.
- Interpretations were excluded from the figures legends.
- The manuscript was read and edited by a native speaker.

The informations requested for Figures 2c and 2d are supplied.
The MM section now contains the information on statistical treatment of the results.

Referee Karunagaran

With respect to minor essential revisions we have corrected the label and supplied the standard deviation in figure 2.
The referee asks why clones 18-2 and 28.8 were not compared in figure 2.

If figure 2c were concerned:
Clone 18-2 did not show any response towards the addition of recombinant TGF-β with respect to cell growth in vitro as measured either in growth curves (2a, b) or through cell cycle distribution (not shown for clone 18-2). Cell cycle distribution of clone 28-8 in response to TGF-β is shown in figure 2c, as growth of this cell clone nearly came to a halt upon long-term exposure to TGF-β.

If figure 2d were concerned:
Clone 18-2 is derived from the parental clone 18, whereas clones 28-8 and 28-14 are derived from parental clone 28. As both parental clones slightly differ in their growth characteristics (see 2a), Smad4-expressing derivative clones need to be compared to their respective parental clone.

The typo on page 11 (now 10) was corrected and all the text was checked for grammatical errors.

Referee Duenas-Gonzalez

The reviewer suggests to perform an immunohistochemical analysis of nude mouse tumors to address the question if BigH3, fibronectin and PAI-1 were in tumor stroma and were responsible for growth delay of tumors treated with doxycycline. This is a very good point, however, we think that it will not be easy to get clearcut results from such analyses:

- **IHC analyses** are inherently difficult to quantify, in particular when matrix components are concerned. The cellular source of the proteins would remain unclear, as also diverse mouse stromal cell types would express the corresponding proteins. So, alterations in cellular constituents of the experimental tumors would have to be addressed in detail. (No gross differences are apparent upon microscopic H&E examination). Moreover, it is not apparent how the description of expression patterns of the three proteins could bring light into the question about their causal relevance for Smad4-dependent growth delay. This would require more focussed analyses like knockdown of each individual Smad4 target followed by the analysis of functional consequences. This, clearly, is beyond the scope of this manuscript.
Referee Fleuren

The reviewer has two major concerns about the manuscript:

1. **The authors do not address the question they pose in the abstract**
2. **The inability to block cell growth after re-expression of Smad4 may be related to changes in this particular cervical cancer cell line**

**Ad 1.**
Are loss of growth inhibition by TGF-β and loss of Smad4 independent events in carcinogenesis? This question is addressed and answered in all parts of the manuscript and is nicely and concisely summarized by the reviewer Karlheinz Friedrich (Chapter: General).

**Ad 2.**
The reviewer suggests to expand the study with at least two further cervical cancer cell lines. However, we and others have previously collected many lines of evidence that loss of growth inhibition by TGF-β and loss of Smad4 are independent events in carcinogenesis. Restoration of TGF-β induced growth inhibition was dispensable for Smad4-mediated tumor suppression in a number of cancer cell models, mostly in colorectal and pancreatic cancer cell lines. Here, we add to this collection a cervical cancer cell line, representing a tumor entity in which deregulation of growth control is usually ascribed to the effects of HPV E6 and E7 proteins on p53 and pRB. We also referred to our previous paper, where we showed that the Smad4 status and TGF-β responsiveness do not correlate in a panel of cervical cancer cell lines.

Here, we show Smad4 dose-dependent control of TGF-β target genes associated with the extracellular matrix. Smad4-dependent control of these genes is additionally confirmed in two pancreatic cancer cell lines manipulated to reexpress Smad4 through retroviral transduction (Fig. 6). Ultimately, Smad4 knockdown in a Smad4-positive pancreatic cancer cell line also shows an impact of Smad4 on transcription control of one of these targets (Fig. 7). We do believe that these results strengthen one of the messages of the paper, namely that Smad4 effects on the composition of the extracellular matrix may underlie or at least contribute to its tumor suppressive activity.

**Minor essential revisions**
We have discussed that decreased cell growth in vitro of Smad4 overexpressing cell clones 28-8 and 28-14 presumably is due to artificial and vast overexpression. It is not the focus of this manuscript to unravel (and discuss) (epi)genetic changes other than loss of Smad4 leading to loss of growth inhibitory responses to TGF-β.

The introduction has been shortened by one third.

Sincerely yours

the authors