CELL BIOLOGY

Comment on "Controlling long-term signaling: Receptor dynamics determine attenuation and refractory behavior of the TGF-β pathway"—Smad2/3 activity does not predict the dynamics of transcription

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The transforming growth factor–β (TGF-β) pathway plays a fundamental role in development and disease. Despite its importance, the dynamics of signaling activity downstream of ligand stimulation have remained largely unexplored. The recent study by Vizán *et al.* demonstrates that loss of signaling-capable receptors from the cell surface leads to a refractory period during which cells are incapable of responding to additional signals. In this letter and in our previous work, we show that although receptor dynamics determine Smad2/3 activity, signaling activity at the level of transcription terminates far earlier in a receptor- and Smad2/3-independent manner. Thus, Smad2/3 activity does not reveal the dynamics of transcription, and downstream measures must be examined directly.

We read with interest the study of Vizán *et al*. (*1*) that examines the dynamics of Smad phosphorylation in the HaCaT human keratinocyte cell line downstream of signaling through the ligand transforming growth factor–β1 (TGF-β1). They find that signaling induces a refractory period, during which cells are incapable of responding to further TGF-β signaling and that this refractory period is caused by a loss of receptors from the cell surface. Under continuous stimulation, signaling, as measured by the abundance of phosphorylated Smad2 (pSmad2) in the nucleus, decays over the course of about 8 hours to a level intermediate between peak stimulation and basal signaling levels (Fig. 1A).

Smads are transcription factors, and their key function is to control transcription in response to extracellular signals; however, Vizán *et al*. analyze the response at the level of pSmad2 without examining downstream transcription. Indeed, although pSmad levels may reflect receptor dynamics, they do not necessarily directly reflect gene expression dynamics. For example, we have shown that transcriptional dynamics in both C2C12 and HaCaT cells are adaptive with a shorter time scale (*2*) than the receptor dynamics studied by Vizán *et al*. We found that transcriptional activity returns to baseline levels, whereas Smad2 and Smad3, collectively referred to as Smad2/3, remain active in the cell nucleus. Instead, the transcription rate is mirrored by the localization status of Smad4 (*2*). Thus, it is important to distinguish between the dynamics of receptor activity and the dynamics of transcription because these two measures are regulated with very different time scales.

To examine how closely the receptor dynamics and Smad2 phosphorylation dynamics reported by Vizán *et al*. reflected TGF-β–dependent transcription, we stimulated HaCaT cells with a saturating dose of TGF-β1 (2 ng/ml), the same dose used by Vizán *et al*. The dynamics of Smad2 nuclear accumulation as measured by immunofluorescence are similar to those of pSmad2 measured by Vizán *et al*. (Fig. 1, A and B; compare to Fig. 1A of Vizán *et al*.). Smad2/3 peaks at 1 hour, and then there is some reduction of total Smad2/3 in the nucleus; however, both the levels of nuclear Smad2/3 and the fraction of total Smad2 that is nuclear remain well above the baseline at all time points examined. The single-cell response was uniform over the field of cells (Fig. 1A). We then examined the transcriptional output from the TGF-β pathway using HaCaT cells with a stably incorporated reporter for TGF-β–mediated transcription (CAGA-luc). To measure when signaling-dependent transcription terminated, we added the Alk5 inhibitor SB431542 (*3*) at various times after TGF-β stimulation and then collected all the samples together after 12 hours, following the technique described in (*4*). The results show that despite the continued presence of nuclear Smad2/3, transcription did not continue to increase, which we interpret as termination of signaling-dependent transcription (and translation) after 2 hours (Fig. 1C). We previously reported quantitative, temporal analysis of the transcript abundance for several Smad-dependent target genes, which showed a similar duration of signaling-dependent gene expression (*2*). Thus, transcriptional output from the pathway is nearly perfectly adaptive with a time scale of about 2 to 4 hours, whereas activated Smads remain in the nucleus for far longer than this.

The relationship of the refractory period observed by Vizán *et al*. to previous studies of repeated exposure to TGF-β is also in need of clarification. The authors estimate that cells need 12 to 24 hours to recover from ligand exposure. In another study (*5*), we found that using microfluidics to repeatedly expose C2C12 cells to TGF-β generated a full and independent response with each exposure, even with only 6 hours between the pulses. Although this may reflect differences between cell lines, another study in HaCaT cells found that cells are capable of responding to repeated pulses of TGF-β separated by as little as 1 hour (*6*) (see Fig. 3 of Zi *et al*., where the TGF-β concentrations tested included some of those comparable to those used by Vizán *et al.*), which is not consistent with the refractory period that Vizán *et al*. describe. Further work will be necessary to resolve these discrepancies.

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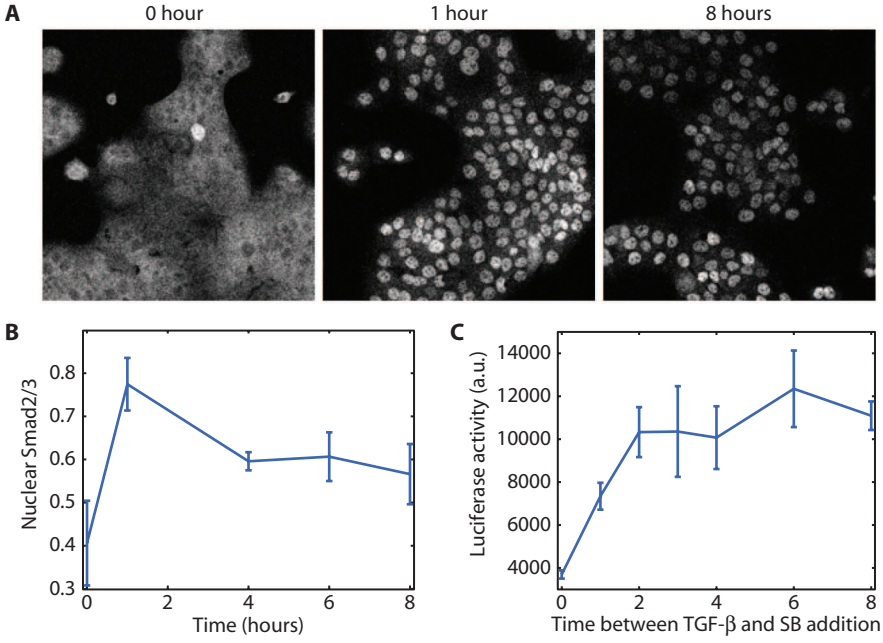

Fig. 1. Smad-dependent transcription is adaptive, whereas the abundance of nuclear Smad is not. (**A**) Images of HaCaT cells stimulated with TGF-β1 (2 ng/ml) for the time indicated. (**B**) Quantification of the experiment shown in (A). Cells were identified using a SYTOX Green counterstain, and the amount of Smad2/3 in the nucleus of each cell was determined and normalized to the SYTOX intensity. The average amount of Smad2/3 in the nucleus is shown. Error bars are the SD from five different images. This experiment was repeated twice. (**C**) Quantification of luciferase activity from cells treated with TGF-β1 (2 ng/ml) and then with 10 μM SB431542 (SB) at the indicated time afterward. All samples were collected together 12 hours after the initial TGF-β1 treatment. a.u., arbitrary units.

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