

Response to Reviewer 1

The paper by Knight et al describes that genetic ablation of the intracellular portion of tissue factor or of PAR2 downstream to tissue factor both reduce in a non-additive fashion fibrosis in the liver induced by treating mice with CCL4. Some insights in the mechanism of action of the two manipulations were provided by histological data indicating that they both reduce the frequency of activated smooth muscle cells and activated macrophages and the levels of TGF-beta, a known profibrotic factor, in the liver. The results are interesting in view of the fact that liver fibrosis is an unmet clinical need which involves a large number of patients and that drugs targeting tissue factor have been developed and are currently in clinical trials for treatment of thrombosis.

Major Comments 1) Although the results were obtained on a robust number of mice, it is unclear how many times the experiments were repeated.

Experiments were repeated in triplicate in each group

Also, only male mice were investigated. Given the gender-biased effects of TGF-beta, the experiments should be repeated also on female mice.

Male mice were chosen precisely to decrease any influence of gender on experimental outcomes; this is a common approach to reduce variability in basic scientific studies. While we agree that clinical studies should include males and females, we also note that TGFβ polymorphisms, rather than gender, influence differential outcomes in fibrosis (Hepatology 2000;31:828-833, J Korean Med Sci 2010; 25: 564-9).

2) Representative histological data should be presented. In addition, the computer method used for their quantification requires a better definition. What HPF means in Figure 2? Is this the same than hpf used in the result section?

Histological data has been added (revised Figure 1). The imaging software is Scion Image for Windows; we used version vAlpha 4.0.3.2. The software is available from Scion Corporation, Frederick, MD. We have used this software in previous publications (Cell Transplantation, Vol. 19, pp. 1157–1168, 2010; Hepatology 2012;55:879-887). HPF is a standard abbreviation for 'high power field'

3) The data are counterintuitive and provide limited mechanist insights. In fact, both deletion of the intracytoplasmic domain of tissue factor, which supposedly activated PAR2 because it is a PAR2 inhibitor, and PAR2 deletion prevented development of liver fibrosis. The authors should check the baseline levels of PAR2 in the tissue factor deficient mice to address this contradiction.

Our results are consistent with others who have shown that the TF cytoplasmic domain is necessary for PAR-2 regulated inflammation; therefore its deletion inhibits PAR2 mediated inflammation (neutrophil activation). (Redecha P, et al. Journal of Clinical Investigation 2008;118:3453 – 3461).

In addition, they should take into consideration that the intracellular domain of tissue factor may repress liver fibrosis by activating an alternative pathway that is dominant over that of PAR2. This dominance may also explain why it is not possible to suppress further liver fibrosis by combining tissue factor and PAR2 deletion.

While the reviewer suggests an interesting alternative explanation for our findings, we are not aware of the existence of an alternative pathway regulated by the TF cytoplasmic domain that would overshadow PAR2 activity.

The hypothesis of two distinctive pathways would also explain the additional paradox mentioned in the paper. That is that deletion of the entire tissue factor protein, by activating thrombosis, induces heart fibrosis, while deletion of the sole intracellular portion of the protein reduced liver fibrosis (this manuscript). Overall

however, I suggest deleting the discussion about heart fibrosis. Fibrosis in the liver and in the heart may be determined by distinctive mechanisms anyhow.

We agree with the Reviewer regarding the possibility of distinctive mechanisms in regard to the heart and the liver and have thus deleted the discussion regarding cardiac fibrosis

4) The authors should carefully revise the manuscript to avoid over-interpretation. As an example, on page 13, line 24, the authors state that they have shown a reduction in macrophage recruitment in the liver of knock out mice following induction of liver fibrosis. Since the livers were stained only with an antibody that identified activated macrophages, the paper does not provide any data on the frequency of the total macrophage population in this organ.

The discussion has been modified to state that we have shown a reduction in recruitment of activated macrophages (CD86+) rather than implying changes in the total macrophage population.

The discussion few lines below on PDGF-BB induced chemotaxis is also not supported by determinations of PDGF-BB expression.

The discussion regarding PDGF has been deleted

5) Efforts should be made to reduce the extensive speculations made in the Discussion. A way to achieve this would be to move the first paragraph of the discussion which provides the rationale for the study in the Introduction and to delete the lines 5-8 on page 14 which deal with breast cancer and not with fibrosis.

We have changed the Discussion as suggested by the Reviewer.

6) Ablation of tissue factor and PAR-2 reduces but does not completely prevent development of liver fibrosis in this model. The absence of data indicating that these levels of reduction have therapeutic significance is a major drawback of the paper.

Our study showed fibrosis reduction in a well-characterised murine model. There are no studies of anti-fibrotic therapy that shown complete prevention of liver fibrosis in either experimental models or in humans. Showing therapeutic significance, which we assume means clinical significance, would require a large scale, long-term human study. While such a study would be of great interest, it is beyond the scope of this manuscript.

Also unclear is whether it is possible to achieve by pharmacological means levels of tissue factor or PAR-2 inhibition similar to those achieved by deleting the genes. Pharmacological inhibitors of TF should not only be discussed but also investigated in this model.

Our study provides proof of concept that PAR2 and the TF cytoplasmic domain influence hepatic fibrogenesis. While it would be of interest to test pharmacological TF inhibitors, those agents are not available to us and therefore were not tested.

Minor comments 1) Figure captures. All the Figure captures shou

These comments are incomplete and therefore we are unable to respond to them