Format for ANSWERING REVIEWERS

July 23, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 20242-Review.doc).

Title: Connective tissue growth factor differentially binds to members of the cystine knot superfamily and potentiates platelet-derived growth factor-B signaling in rabbit corneal fibroblast cells

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The following is our point-to-point rebuttal based on four reviewers' comments. These comments are listed and italicized to distinguish authors' responses for the convenience of reading as below.

Comments from the reviewer 01404215: *"The manuscript is clear, well written and complete. The antagonism for the binding between CTGF and the receptors of several growth factors is clearly explained and shows the basis of CTGF action to regulate angiogenesis and fibrosis at specific moments of animal life, for example during embryo development."*

Response: We thank the reviewer's positive comments.

Comments from the Reviewer 02557824: *"The topic of this work is of high interest. The authors approach to the study of the interactions between CTGF and a number of its interactors using the LexA-based yeast two-hybrid system and SPR analysis, and tried to explain the results using some functional aspects. I suggest to accept with major revisions. Because there are some points unclear, in particular: A) the measured KD of 43 nM for the interaction between PDGF-B and CTGF by SPR analysis is not in agreement with the data*

obtained for LexA-based yeast two-hybrid experiments, in fact TGF-β1 that in figure 1 show a better affinity toward CTGF, has a comparable KD between 30 and 60 nM. Please clarify this point."

Response: A) Measurement of relative β-galactosidase activity showed that cells containing the cystine knot of TGF-β1 had a 3.80 ± 0.66 fold increase and the ones with the cystine knot of PDGF-B had a 2.64 ± 0.33 fold elevation in LexA-based yeast two-hybrid experiments (Figure 1). These observations indicated that CTGF binding to TGF-β1 was stronger than to PDGF-B. SPR analysis detected that PDGF-BB and CTGF had a dissociation constant (Kd) 43 nM (Figure 2). A lower dissociation constant (30 nM) was reported by Abreu et al,[1] supporting our observation that CTGF had a higher affinity to TGF-β1 than to PDGF-B. Recently, Khattab et al have reported that CTGF has a dissociation constant 64.7 nM for TGF-β1 and 230 nM for PDGF-BB.[2] These dissociation constants are varied in comparison to ours and Abreu et al, likely due to utilization of different Biacore systems. For examples, Khattab et al immobilized CTGF on C1 sensor chips for TGF-β1 and CM5 sensor chip for PDGF-BB, whereas we cross-linked CTGF on CM4 dextran sensor chips. In contrast, Abreu et al ran CTGF protein over CM5 sensor chips that were coated TGF-β1. Nevertheless, all of the dissociation constants indicate weak interaction in nM range. Our findings are consistent with studies from Khattab et al and indicate that CTGF has relatively lower affinity to PDGF-BB than to TGF-β1.

"B) The amount of PDGF-B in the PDGF-B/PDGFRβ experiments in presence of CTGF is very low with respect to the amount of CTGF, how explain the authors the continued rise of the signal with the addition of CTGF without the reaching of a plateau?

Response: We mixed 2 nM PDGF-BB with 0-263.2 nM CTGF and tested whether CTGF influenced the binding of PDGF-BB to PDGFRβ immobilized surface in SPR analysis in Figure 3B. CTGF at concentrations that were more than 26.3 nM could significantly enhance the binding of PDGF-BB to PDGFRβ. A dose dependent binding of PDGF-BB to PDGFRβ was also observed when the concentration of CTGF increased to 263.2 nM, but a plateau was not observed during tested concentrations of CTGF. This is due to usage of a low concentration of PDGF-BB (2 nM) in this experiment and a weak interaction between CTGF and PDGF-BB we detected. Higher concentration of CTGF appears to be required to reach the plateau of PDGF-BB and PDGFRβ binding.

"C) In the SPR analysis the author not exclude the possibility of a specific or nonspecific interaction of CTGF to the PDGF-B/PDGFRβ complex, probably new experiments should be carried out in order to demonstrate the obtained results."

Response: CTGF was initially identified using anti-human PDGF IgG affinity chromatography.[3] Direct physical interaction between CTGF and PDGF-B was supported in our yeast two-hybrid analysis[4] and SPR analysis (Figure 1 and 2B). Another independent study from Khattab et al indicates that CTGF directly interacts with PDGF-BB in SPR analysis. In addition, we demonstrated that CTGF protein in a high concentration (263.2 nM) was not able to interact with PDGFRβ in SPR analysis (supplemental Figure 2). Literatures and our findings strongly suggest that CTGF specifically interacts with PDGF-B but not PDGFRβ.

"D) Conclusions are very generic and lacking in the description of a clear mechanism of action, functional data do not support the other results so this part must be improved."

Response: Revised conclusion for Figure 3 is "These results indicated that CTGF could potentiate some PDGF-B signaling including activation of PDGFRβ and AKT in rabbit corneal fibroblast cells".

Minor revision:

"1) The authors forgot to indicate the figure 1A in the text."

Response: Figure 1A has been added in the revised text.

"2) In figure 2A there are 6 lines and 5 concentrations indicated (red line)."

Response: We are sorry for this confusion. This extra red line is removed in revised Figure 2A.

"3) In figure 2B the concentrations of PDGF-B are different from the ones reported in the figure legend as well as in the text (the concentrations refer to the amount of PDGF-B in figure 3A)"

Response: Correct concentrations of PDGF-BB ranging from 19.3 nM to 385 nM have been indicated in the text and in revised Figure 2B.

"4) Authors move from the use of nM (more precise) to the use of ng or ug/ml, this unit of measure avoid to comparethe results with the previous experiments. I suggest to use only molar concentration in all paper for a better comparison of data."

Response: Changes for molar concentration has been made in all of the paper.

"5) Higher concentrations of CTGF are indicated to be used in materials for the PDGF-B/PDGFRβ experiments, but results are not showed."

Response: We used 10 μg/ml (263.2 nM) in this study, however, this was labeled as 5 μg/ml by mistake. We are sorry for this confusion. Correct label for CTGF concentration has been shown in text and Figure 3B of the revised manuscript.

"6) It is unclear if the authors use PDGF-B and PDGF-BB to describe the use of domain, monomer or dimer of PDGF-B, please make order on this."

Response: PDGF-B is used to described gene or protein. PDGF-BB is used to describe the homo-dimer, an active form of PDGF-B protein that is recognized by its cognate receptor PDGFRβ.

"7) The controls in the interaction experiments between PDGF-B/PDGFRβ in presence of CTGF are important to demonstrate the absence of the direct interaction between PDGFRβ and CTGF, so please add as supplementary data."

Response: The control experiment has been added as Supplementary Figure 2 in the revised manuscript.

"8) Raw data for the elaborations in figure3B referring to PDGF-B/PDGFRβ interaction in presence of increasing CTGF concerntrations, should be added as supplementary data."

Response: Raw data for Figure 3B have been shown as new Supplementary Figure 1.

Comments from the Reviewer 02557824: *The manuscript by Pi et al. compared binding of CTGF to several cycstine knot proteins including VEGF-A, PDGF-B, BMP4 and TGF-beta1. Binding data are consistent in two systems: yeast two hybrid and SPR. The binding to PDGF-B was shown to enhance its association with its receptor and the activation of downstream signaling. Some minor comments: The name PDGF-B was used in the yeast two hybrid study, but PDGF-BB was used in SPR and cell signaling assay. Are they referred to the same protein? Is there any reason that two different names should be used in the same paper?*

Response: We thank the reviewer's positive comments. PDGF-B is used to described gene or protein. PDGF-BB is used to describe dimer form of PDGF-B protein.

Comments from the Reviewer 01585205: *CTGF is known as an important growth factor in regulating diverse biological functions, including cell adhesion, migration, tissue wound repair, fibrotic disease cancers. In this paper, the author demonstrated that CTGF has different binding strengths to VEGF-A, PDGF-B, BMP-4, and TGF-β, which regulate these growth factors triggered downstream signaling pathway. The paper is well written. The data presented are clean with appropriate controls, the experiments are outlined clearly and in logical order. Additional comments as detailed below.*

"1. PDGFR can bind with multiple tyrosine kinase through its SH2 and SH3domain, the author proved that CTGF can influence PDGFR-mediated PI3K-AKT activation. Can CTGF also influence other PDGFR-mediated signalling pathway?"

Response: 1. So far, we only found that CTGF influenced PDGF-B stimulated AKT phosphorylation. Future studies will determine whether CTGF influences other PDGFR-mediated signalling pathways.

"*2.Why CTGF have no impact on PDGFR-triggered ERK activation?"*

Response: Activation of signal transduction pathways depends on the amount of ligands, the expression and cell surface localization of their receptors, and the expression of receptor family members as well as

various docking proteins. PDGF-B is a growth factor and forms an active homo-dimer that binds its cognate receptor PDGFRβ leading to downstream signal transduction. In contrast, CTGF can elicit adhesive signaling through various types of cell surface receptors including integrins, heparin sulfate proteoglycans (HSPGs) and low density lipoprotein receptor related proteins (LRPs).[5-8] In this paper, We found that CTGF was able to potentiate PDGF-BB binding to PDGFRβ and enhance the phosphorylation of PDGFRβ and downstream AKT molecule, even though CTGF had no impact on PDGFRβ-triggered ERK1/2 activation. Considering that synergy exists between integrins and PDGFRβ,[9] we believe that CTGF and PDGF-B binding is part of crosstalk between the two pathways and contributes to the enhanced activation of AKT, whereas no merge of the two pathways in certain downstream signaling may account for the lack of enhancement of ERK1/2 activation in cultured rabbit corneal fibroblast cells. Future studies about interplay between CTGF/integrins and PDGF-B/PDGFRβ signaling are needed to clarify the nature of CTGF enhanced PDGF-B signaling. These explanations have been incorporated in Discussion Section in the revised manuscript.

References

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