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Thymosin β4 in Vascular Development

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To the editor,

We thank you for the opportunity to respond to the commentary from Banerjee et al, regarding our publication "Essential Role for Thymosin β4 in Regulating Vascular Smooth Muscle Cell Development and Vessel Wall Stability."

The discrepancies between our findings and those of Banerjee and colleagues were outlined in our original paper[1] and plausible explanations provided, which were acceptable to the reviewers of our manuscript. We reiterate below our rationale and data that support a role for Tβ4 in mural cell development (in addition to those presented in our original publication). We did not, as stated in the Banerjee commentary, report impaired mural cell migration.

- We demonstrate consistency of phenotype between our knockout and knockdown model; defective smooth muscle differentiation in global Tβ4 KO mice (systemic and coronary) is also observed in the systemic vasculature of Tie2Cre-Tβ4shRNA mice and in the coronary vasculature of Nkx2-5Cre-Tβ4shRNA. The probability of faithfully recapitulating an "off-target" phenotype in an shRNA-based knockdown model by "perturbation of Tβ4-independent events" in a global knockout would be low.
- The roles inferred for Tβ4 from our study are entirely consistent with the previously reported role for Tβ4 in vascular smooth muscle cell differentiation, determined by gain-of-function analyses in multiple tissues and from several laboratories: in the developing yolk sac[2], the coronary vasculature after injury[3-6], in addition to the *in vitro* differentiation of A404 progenitor cells described within our paper (in which Tβ4 promotes vascular smooth muscle cell (VSMC) fate).
- Further compelling evidence for Tβ4 knockdown, and not an off-target effect, as the primary cause of the phenotype comes from the demonstration that severity of phenotype correlates with the extent of Tβ4 knockdown[3].
- We acknowledge the risk of off-target activity and the inadequacy of screening techniques to exclude such a possibility. We have, however, taken reasonable steps to obviate off-target activity. Jackson and Linsley[7] advocate multiplicity (the use of multiple individual siRNAs targeting the same gene, since each siRNA has a unique off-target spectrum, but the same intended target) to mitigate against off-target siRNA. To this end, we previously tested two shRNAs against Tβ4[8], both of which disrupted actin filament formation and decreased motility in NIH-3T3 fibroblasts (unpublished), increasing confidence that the phenotype results from silencing the intended target. We additionally

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demonstrated the lack of Tβ10 knockdown, the most closely related gene, additionally supporting selectivity of Tβ4 knockdown[3, 8].

- We partially characterised the mechanism by which surviving Tβ4 KO embryos compensate for loss of Tβ4, namely the hyper-activation of TGFβ (Smad) signalling. This is in keeping with our demonstration that Tβ4 synergistically activates TGFβ signalling and further supports a *bona fide* knockout phenotype resulting from impaired Tβ4-mediated signalling.

Banerjee and colleagues present further data (immunohistochemical analysis of developing aorta and survival data) in attempt to reaffirm their claim that Tβ4 is not required for vascular development. The immunohistochemical data, upon which the authors conclude that mural cell coverage is not impaired, differs considerably from that presented in their original publication. The original images (Figure 2C and D, page 459) unintentionally revealed a mural cell defect identical to that which we demonstrate[9] and the authors now state that these panels “were meant to illustrate maintenance of vascular smooth muscle within the aorta and were not meant to be a quantitative assessment”. Further oversights confound interpretation of the new data, specifically:

- The figures presented to contradict our findings consist of immunofluorescence on Tβ4 global KO aorta from E12.5-E14.5 embryos. In order to provide a direct comparison, the authors should analyse mural coverage of the aorta at E10.5, the time point at which we observed significant reduction in mural cell coverage. By E14.5 our severe mutants are dead and only compensated mutants with no haemorrhagic phenotype survive beyond E10.5 (hyperactivation of TGFβ signalling by an alternative pathway permits normal mural cell differentiation). Although Banerjee et al report no lethality, any delay in mural cell differentiation in their knockout line may be overlooked by analysis at these later time points.

- The authors have now examined endothelial-specific Tβ4 knockout embryos, yet no data on vascular histology or mural cell differentiation are presented. Moreover, the authors claim a lack of lethality in this strain. While lethality is not statistically significant based on the sample size, the trend reported is consistent with a preferential loss of mutant embryos.

- PDGFRβ, although expressed in some pericytes, is a poor choice of mural cell marker in this context (Figures 1 and 2). PDGFRβ is expressed in undifferentiated periaortic mesenchyme but down-regulated upon differentiation into VSMCs, as illustrated by Shinbrot et al[10]. We believe this may account for the apparently higher expression of PDGFRβ in the adventitial/periadventitial layers than in medial VSMCs, as evident in both figures of the commentary. We examined a range of pericyte and VSMC markers quantitatively (both by qRT-PCR and by Image J analysis on directly comparable images from multiple embryos) and found NG2, SMαA, SM22, SM-MHC, endosialin, CD13 and angiopoietin 1 to be significantly down-regulated in severe Tβ4 KO embryos.

Thus, we explain the discrepancies between our data and those of Banerjee as follows:

- A variable extent in both requirement and capacity for induction of a compensatory mechanism following global knockout versus excision of a floxed allele or induction of shRNA expression. It is clear that global Tβ4 knockout mice efficiently employ a compensatory mechanism in order to survive (100% of the Banerjee mice and 80% of our mice). The many G-actin sequestering proteins, including other thymosin family members, could compensate and, in the context of mural cell differentiation, multiple pathways feed into TGFβ

signalling to mediate compensatory activation. Indeed, we confirmed this supposition in our study. Complete cellular knockout, even in a cell type-specific context such as the Nkx2-5Cre or Tie2 Cre-driven KOs created by Banerjee et al, could also evoke such compensatory mechanisms. We have been unable, using shRNA, to achieve complete knockdown of Tβ4 and incomplete knockdown either does not require or does not sufficiently trigger compensatory pathways.

- Our careful analyses of global and endothelial Tβ4 loss-of-function models, over the time-course of development, using multiple markers and a large number of litters, allows us to confirm a role for Tβ4 in mural cell differentiation and vascular development. Such a role is consistent with the demonstrated gain-of-function role of exogenously administered TB4 in promoting smooth muscle differentiation. The demonstration that loss of Tβ4 can be compensated for does not preclude a role for Tβ4 to ordinarily function in such a context.
- Genetic background or environmental differences may contribute towards an exacerbated KO phenotype in our hands but do not detract from the findings, since littermate control embryos were derived on the same genetic background and environment.

We therefore refute the claims of Banerjee et al. and maintain our assertion that Tβ4 ordinarily participates in mural cell differentiation for development of a stable, functional vasculature.

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