Thymosin β4 Is Not Required for Embryonic Viability or Vascular Development

Indroneal Banerjee, Thomas Moore-Morris, Sylvia M. Evans, Ju Chen

**Rationale:** Rossdeutsch et al describe a requirement for thymosin β4 (Tβ4) in vascular development. Impaired mural cell migration, differentiation, partial embryonic lethality, and hemorrhaging were observed after analysis of 2 lines of mice, one of which was germline null for Tβ4 and another in which Tβ4 was knocked down by endothelial-specific expression of Tβ4 short hairpin RNA. These data are in direct contrast to our published global and cardiac-specific Tβ4-knockout lines. Thus, the role of Tβ4 needs to be clarified to understand its importance in cardiovascular development.

**Objective:** To investigate and clarify the role of Tβ4 in vascular smooth muscle cell development and vessel stability.

**Methods and Results:** Examination of Tβ4 global knockouts did not demonstrate embryonic hemorrhaging, altered mural cell development, or lethality. Endothelial-specific knockouts also did not exhibit any embryonic lethality and were viable to adulthood.

**Conclusions:** Analysis of our Tβ4 global and cardiac- and endothelial-specific knockout models demonstrated that Tβ4 is dispensable for embryonic viability and vascular development. (Circ Res. 2013;112:e25-e28.)

**Key Words:** cardiac development ■ thymosin β4 ■ vascular biology ■ vascular smooth muscle

Understanding signaling factors that regulate the formation of the vasculature can lend significant insight into both development and disease. Thymosin β4 (Tβ4) is a 43-aa factor initially found to interact with G-actin and regulate F-actin formation.1 Recent studies have indicated that administration of Tβ4 to either postschemia reperfusion or myocardial infarction models can improve cardiovascular function and abrogate scar formation, primarily through de novo vascular formation.2–4 Early studies using a short hairpin RNA (shRNA) knockdown approach suggested that Tβ4 acted as a key regulator of blood vessel formation (angiogenesis, vasculogenesis, and arteriogenesis).5 However, our studies using both global and cardiac-specific knockout approaches found Tβ4 to be dispensable for embryonic viability and vessel development.6

---

**See Response, p e29**

In a recent article, Rossdeutsch et al7 describe a requirement for Tβ4 in vascular development. Impaired mural cell migration and differentiation were observed after analysis of 2 lines of mice, one of which was germline null for Tβ4 and another in which Tβ4 was knocked down by endothelial-specific expression of Tβ4 shRNA. Rossdeutsch et al7 report that global knockout of Tβ4 resulted in partial lethality at the start of the study that was decreased in subsequent generations later during the study, an incompletely penetrant hemorrhagic phenotype, and decreased mural cell coverage of the aorta. Tβ4 knockdown in endothelium also resulted in partial lethality and decreased mural cell coverage.7 These data are in direct contrast to our published Tβ4-knockout model.6 Thus, to clarify the role of Tβ4 in vascular formation, we further analyzed our global knockouts of Tβ4 and an endothelial-specific Tβ4 knockout not previously reported. Consistent with our previous results, we did not observe an evident phenotype, alteration in vascular development, or embryonic lethality. We conclude from these data that Tβ4 is not required for embryonic viability or vascular development.

**Materials and Methods**

**Animal Care**
All animal procedures were performed and approved by the University of California, San Diego Animal Care and Use Committee.

**Generation of Tβ4 Floxed, Knockout, and Endothelial-Specific Knockout Mice**
Tβ4-targeted mice were generated and used as previously described to a C57/B6J background.8 Endothelial-specific knockout mice were generated by crossing Tβ4−/− female mice to Tie2-Cre male mice.

**Immunofluorescence Analyses**
Frozen sections from embryonic day (E) 14.5, E13.5, and E12.5 embryos were isolated and stained as previously described.6

Original received October 5, 2012; revision received November 12, 2012; accepted November 21, 2012. In December 2012, the average time from submission to first decision for all original research papers submitted to Circulation Research was 15 days.

From the Department of Medicine (I.B., S.M.E., J.C.) and Skaggs School of Pharmacy and Pharmaceutical Sciences (T.M.M., S.M.E.), University of California-San Diego, La Jolla, CA.

Correspondence to Ju Chen, Department of Medicine, University of California San Diego, 9500 Gilman Dr, La Jolla, CA 92093. E-mail juchen@ucsd.edu

© 2013 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

**DOI:** 10.1161/CIRCRESAHA.111.300197
Results

Mural Cell Coverage and Development Are Not Altered in Global Null Tβ4 Mutants

To clarify the role of Tβ4 in large vessel development, we examined our global Tβ4-knockout mice from E12.5 to E14.5 (Figures 1–2). Initial observation in these stages revealed no gross abnormalities or hemorrhage between wild-type and knockout samples. To examine large vessel defects, we first examined aortas in E14.5 embryos (Figure 1). At this stage, no differences were observed between examined wild-type and knockout embryos. To rule out developmental and mural cell recruitment defects, E12.5 and E13.5 aortas were stained with antibodies to α-smooth muscle actin and platelet-derived growth factor subunit-β, 2 markers of vascular smooth muscle cells (Figures 1 and 2).6–8 No differences were observed between control and Tβ4 null embryos.

Endothelial-Specific Deletion of Tβ4 Does Not Result in Embryonic Lethality

Rossdeutsch et al7 observed 12% lethality in embryos with endothelial-specific shRNA-mediated knockdown of Tβ4. As stated in previous publications, shRNA knockdown can have off-target effects.6,9 To further investigate this possibility, we used the same Tie2-Cre used by Rossdeutsch et al.7,10 Our Tβ4f/f females6 were bred to Tie2-Cre male mice.10 Male Tie2-Cre mice were also used by Rossdeutsch et al in their knockdown studies. Of the 34 postnatal male mice, 18 were Tβ4f/f and 16 were Tβ4f/f;Tie2-Cre+, demonstrating lack of embryonic lethality in endothelial-specific knockouts of Tβ4. These data are similar to our global and cardiac-specific knockouts of Tβ4,6 where we did not observe any postnatal lethality or vascular phenotype.

Discussion

Rossdeutsch et al7 report that global Tβ4-knockout mice present with hemorrhage and defective mural cell recruitment, resulting in partial lethality between E10.5 and E14.5. These data are in direct contrast to our published global Tβ4-knockout model.6 In the Discussion section of their article, Rossdeutsch et al refer to our article6 and state that, from our data, “Tβ4 knockout aortas had an apparent reduction in α-SMA+ cell coverage within the vessel wall relative to wild-type controls at E14.5.”7 We believe this to be a misinterpretation of our data. In our publication6 we did not report, nor observe, differences in mural cell coverage in any of our samples at E14.5. Data showing α-smooth muscle actin staining of aortic vascular smooth muscle were meant to illustrate maintenance of vascular smooth muscle within the aorta and were not meant to be a quantitative assessment. However, given the observations by Rossdeutsch et al, we have specifically examined aortas in multiple sections from E12.5 to E14.5 Tβ4 null and control embryos and found no differences between control and Tβ4 null embryos (Figures 1 and 2). We also did not observe any hemorrhaging in any of the embryos in our studies. Thus, our data do not support an essential role for Tβ4 in vessel development.

Rossdeutsch et al7 also suggest that differences in observed phenotypes between our Tβ4 null mutants and theirs can be attributed to either strain background–dependent effects or distinct gene targeting strategies. Initially, Rossdeutsch et al7 observed 40% lethality of their global null Tβ4 mice in a mixed C57/B6J and 129Sv background, with reduction to 20% lethality when bred into a pure C57/B6J background.
However, our global null mice were bred into a C57/B6J background and yet did not display any phenotype. It is possible that differences in observed phenotypes between the 2 Tβ4 null mutant lines may result from variables, such as environment, diet, and infectious state, which may differ between the 2 mouse facilities.

An alternative explanation to strain- or facility-dependent phenotype is that the targeted embryonic stem cells used to establish the Rossdeutsch mutant mouse line contained Tβ4-independent lethal mutations that may have been bred out over successive generations. Furthermore, the possibility raised by Rossdeutsch et al7 that differences in targeting strategies, in independent lethal mutations that may have been bred out over successive generations. Furthermore, the possibility raised by Rossdeutsch et al7 that differences in targeting strategies, in particular the retention of the Neo cassette in the Rossdeutsch et al targeted allele, could also account for discrepant phenotypes is a possibility. However, if this is the case, it is unlikely to reflect differences in targeting Tβ4 function, as exon 2, encompassing 75% of the coding sequence, is deleted in both knockouts and similarly Tβ4 protein is absent, as demonstrated by protein analysis, in both knockouts. Therefore, if the different phenotypes are a result of retention of the Neo gene in the Rossdeutsch Tβ4 null allele, observed phenotypes may result from non-Tβ4-related functions.

Rossdeutsch et al7 argue that there are no off-target effects with their shRNA gene silencing approach and state that “the differences between our global knockout and knockdown model are probably attributed to the fact that RNAi targeting in vivo, when sufficiently optimal to abrogate expression of the target gene, can result in a more severe phenotype than a corresponding global-null. Genetic ablation via homologous recombination through the germline, leading to complete loss of function from the outset in development, may be partially compensated for by functional orthologues, whereas RNAi-mediated efficient knockdown, occurring rapidly and at a defined developmental stage, may not be permissive for compensation.” Our previously published cardiac-specific (Nkx2.5-Cre, αMHC-Cre)6 and endothelial cell–specific (Tie2-Cre; reported here) ablations of our Tβ4 floxed alleles have demonstrated that targeted deletion during development does not result in an evident phenotype, thus calling into question this argument by Rossdeutsch et al to address discrepancies between their global Tβ4 null and their conditional shRNA-Tβ4 knockdown phenotypes.

In summary, our data with global and conditional Tβ4-knockout mice suggest that the shRNA knockdown approach used by Rossdeutsch et al may have off-target effects that result in observed phenotypes. The phenotype observed in their global Tβ4-knockout mice may result from perturbation of Tβ4-independent events. Together, these data do not support an essential role for Tβ4 in vessel development or embryonic viability.

Sources of Funding

J. Chen and S.M. Evans are funded by grants from the National Heart, Lung, and Blood Institute. J. Chen is the American Heart Association (AHA) Endowed Chair in Cardiovascular Research. T.M. Morris and I. Banerjee are supported by AHA postdoctoral fellowships (11POST7310066 and 12POST12030256). Microscopy work was performed at the UCSD Neuroscience Microscopy Shared Facility and was supported by the National Institutes of Health (grant P30 NS047101).

Disclosures

None.

References


Thymosin β4 Is Not Required for Embryonic Viability or Vascular Development
Indroneal Banerjee, Thomas Moore Morris, Sylvia M. Evans and Ju Chen

Circ Res. 2013;112:e25-e28
doi: 10.1161/CIRCRESAHA.111.300197
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/112/3/e25

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/