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科学研究費助成事業　　研究成果報告書

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研究課題名（和文）Significance of a troponin complex gene in embryonic development and disease

研究課題名（英文）Significance of a troponin complex gene in embryonic development and disease

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交付決定額（研究期間全体）：（直接経費）　　2,200,000円

研究成果の概要（和文）：This study analyses how a Troponin complex gene is regulated during embryonic development and is elucidating its unexplored functions in embryonic development and disease.

研究成果の学術的意義や社会的意義
We will study the mechanisms of abnormalities in troponin mutant embryos based on: (1)The defects of sarcomeric Troponin impair skeletal muscle contractility and result in abnormal phenotypes, and (2) The troponin elicits non-sarcomeric actions mainly in the embryonic skeletal muscle.

研究成果の概要（英文）：Components of the Troponin protein complex are involved in the regulation of muscle contraction. However, much is left unknown about possible abnormalities due to their deficiency. This study analyses how a Troponin complex gene is regulated during embryonic development and is elucidating its unexplored functions in embryonic development and disease. we have obtained various data including their phenotypes at the level of skeletal muscle structure, as well as defects of the lymphatic system in embryos.

研究分野：Developmental Biology

キーワード：Troponin

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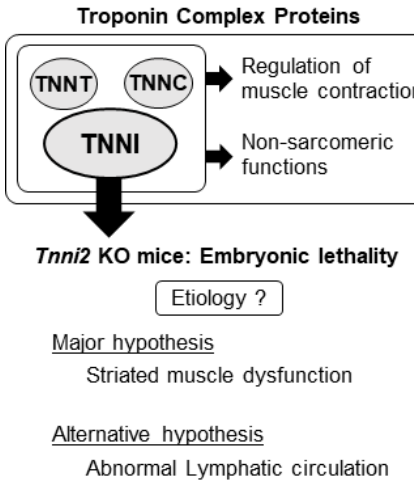
1. Scientific background and key scientific question

Fig 1) General concept of this project



The TNNT, TNNC and TNNI families of Troponin proteins compose a heterotrimeric complex that is fundamental in regulating Ca^{2+}-dependent muscle contraction (Sheng et al., 2016) (**Fig 1)**. In addition to their presence in myocyte thin filaments, the Troponin complex proteins are detectable in the nuclei and other cellular components of various cell types as well as in extracellular vesicles (Johnston et al. Oncotarget 2018).

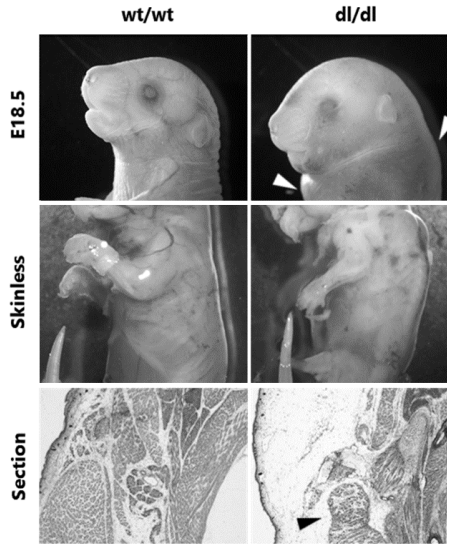
Members of the TNNI genes express an inhibitory subunit of the Troponin complex. Gain-of-function mutations of human *TNNI2* are responsible for Sheldon-Hall syndrome (distal arthrogryposis type 2B) (Toydemir et al., 2009). Details of disease etiologies are still elusive, but insufficient control of muscle contraction likely causes contractures and other muscle/skeletal abnormalities. On the other hand, mounting evidence points to possible involvement of Tnni2 protein in non-sarcomeric biological phenomena including endothelial cell proliferation and tumor growth (Johnston et al., 2018). For example, an inhibitory role on blood vessel growth was proposed for Tnni2, which was mediated by an interaction with the cell surface receptor for basic fibroblast growth factor (Feldman L, et al. 2002).

Despite such importance in health and disease, nothing is known about possible abnormalities due to the *Tnni2* deficiency in humans and other species. We found that *Tnni2* global KO mice showed neonatal lethality due to severe embryonic edema and skeletal muscle abnormalities. We further demonstrated that the myocyte-specific *Tnni2* deletion completely reproduced the global KO phenotypes. In this context, the key focus of the proposed projects is the characterization of embryonic abnormalities in *Tnni2* knockout mice and elucidation of molecular functions of Tnni2 implicated in its KO phenotypes.

2. Purpose, significance and originality of the project

We were able to perform various phenotypic analyses of *Tnni2* global KO mice, which showed severe generalized edema, skeletal muscle disorganization and disruption of the lymphatic system in embryos, followed by respiratory failure and death in newborns (**Fig 2&5&6**). The overarching goal of the current proposal is to explore novel molecular mechanisms of Tnni2 actions focusing on the interesting features of these KO mice. These studies will open up a novel research field to clarify essential Tnni2 functions in embryonic development.

Fig 2) Phenotypes of ***Tnni2*** KO embryos



3. Method of research

Our group has started investigating on Tnni2 by examining its expression in mouse embryos. Immunohistochemistry (IHC) and western blot data using Tnni2 antibody revealed the presence of the protein exclusively in skeletal muscle cells (**Fig 3A & B**) as early as E14.5 (data not shown). To study the outcome of the *Tnni2* deficiency *in vivo*, we generated *Tnni2* KO mouse lines by CRISPR/Cas9 gene editing. Deletion of protein-coding exons resulted in the loss of Tnni2 protein in the skeletal muscle (**Fig 3A**). The obtained heterozygous mice for the deletion (dl) allele grew normally and were fertile but their intercross gave no living homozygous mutant mice after birth (P0.5) (**Fig 4A**). These KO mice appear to die because of severe respiratory failure soon after birth, as showed by lung inflation assessment using the lungs of E18.5 embryos delivered by caesarean section and monitored for 1h during exposure to room air (**Fig 4B**). We then performed some of KO phenotype analyses. In *Tnni2* mutant, massive swelling, which was prominent in the head and upper body, became apparent at E17.5-18.5 (white arrowheads in **Fig 2**). An atrophied body and a decreased total size of muscle groups (black arrowhead) was also noticed. Clear abnormality in myofiber structure and an increase in interstitial space between fibers was seen at E18.5, as shown by both histology and immunofluorescence staining for the muscle cell marker Myosin Heavy Chain (MF20) (**Fig 5**). Hematoma at the upper forelimbs appeared in some embryos with severely affected blood vessel structure marked with CD31. The abnormal enlargement of a subcutaneous layer coincided with significant increase in lymphatic capillaries (white arrowheads) marked with Lyve1 (**Fig 6**).

Fig 3) Tnni2 protein expression check

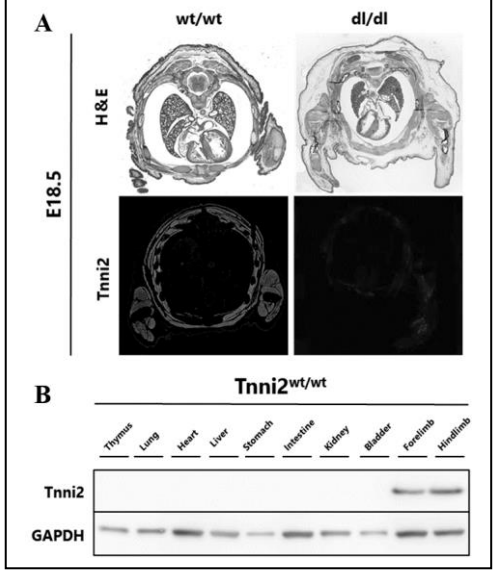


Fig 4) Lethality of ***Tnni2*** KO mice

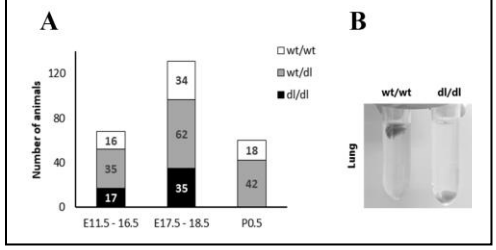


Fig 5) Structural defect in ***Tnni2*** KO striated muscle cells

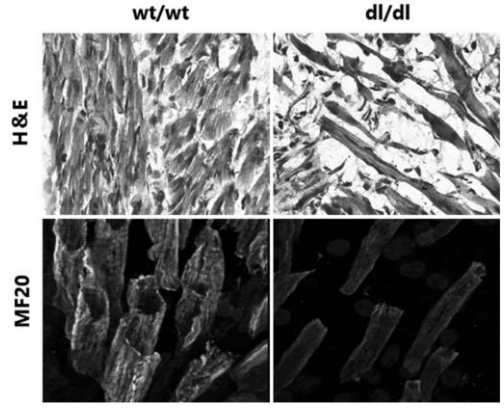
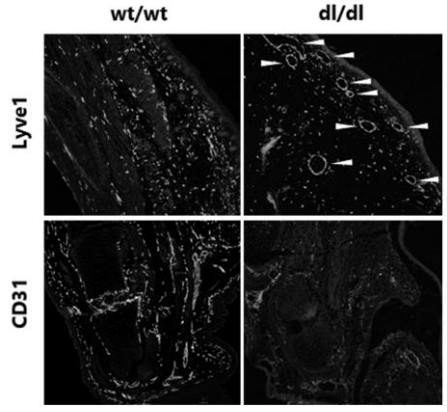


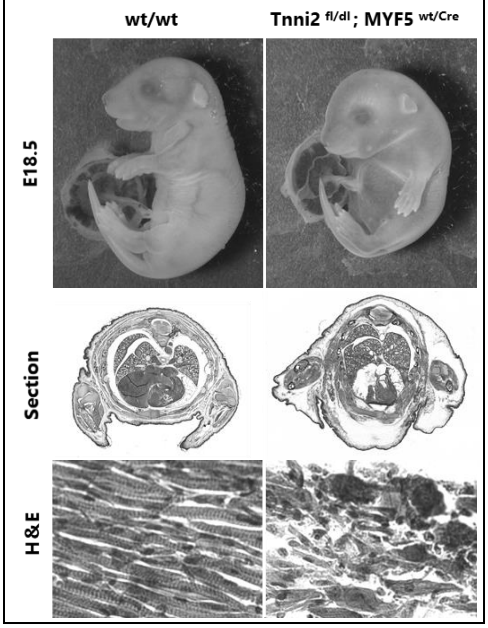
Fig 6) Abnormal embryonic circulation in ***Tnni2*** KO embryos



4. Preparation status towards achievement of the purpose of the research project

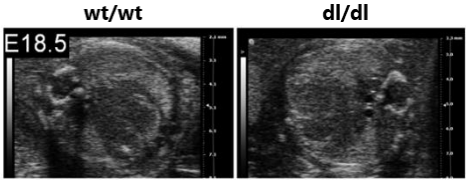
We were able to demonstrate abnormal myofiber structure and an increase in the lumen of initial lymphatics in *Tnni2* KO mouse. To analyze the cell-type specificity of Tnni2 function, we successfully generated *Tnni2* floxed mouse line using CRISPR/Cas9 gene editing then acquired mice expressing Tek-cre targeting endothelial cells as well as Myf5-cre and Myogenin-Cre for skeletal muscle.

Fig 7) Phenotype of ***Tnni2*** conditional KO embryos



Results using Myf5-cre mediated deletion showed the same severe generalized edema as *Tnni2* global KO. We also confirmed the expansion of the subcutaneous layer and defects in myofibers size and structure (**Fig 7**). Mating has already started for Myogenin-cre, and we are expecting similar results as Myf5-cre lines. Although we demonstrated that myofibers are structurally abnormal in all skeletal muscle tissues, *in utero* echocardiography did not indicate cardiac anomalies or impaired contractile function (**Fig 8**). Because some reports demonstrated an expression of Tnni2 in endothelial and vascular smooth muscle cells, and its potential functions in vascular growth factor signaling and hormone-responsive transcription, wedecided to specifically delete *Tnni2* in endothelial cells using Tek-cre mice. Unlike the global KO, we were able to get several living pups and *Tnni2* specific deletion in endothelial cells does not seem to affect the general appearance of mutant embryos, excluding a possibility of endothelial pathologies so far. We plan to keep these healthy looking cKO mice until we confirm they display no abnormalities.

Fig 8) Embryonic echocardiography



５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関