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論文題目	Role of the RNA binding protein Musashi2 in myogenesis		

（論文内容の要旨）

Understanding the regulatory mechanisms of skeletal muscle development and function is vital for keeping and improving human health. Past studies have identified myogenic regulatory factors (MRFs) regulate a cascade of genes for skeletal muscle development at the transcriptional level. Recent studies also discovered that RNA binding proteins (RBPs) play essential roles in the post-transcriptional regulation of myogenesis.

The RBP Musashi2 (Msi2) is a translational regulator of cell fates in normal tissues such as hematopoietic, mammary, and neural tissues, as well as in cancers, including glioblastoma and leukemia. Msi2 is also expressed in normal skeletal muscle tissues, but its role in myogenesis remains largely unknown. This thesis aims to establish a comprehensive understanding of how Msi2 regulates skeletal muscle development and function.

In order to assess the importance of Msi2 in myogenic differentiation, I utilized the C2C12 mouse myoblast cell line as a model. First, I analyzed Msi2 expression during C2C12 differentiation and found that Msi2 is greatly upregulated during the differentiation process. In contrast to a well-characterized role of Msi2 in stemness in hematopoietic or neural lineages, Msi2 is likely to be functional as a differentiation factor in myoblast. Next, to examine if Msi2 is required for this process, I performed knockdown (KD) of Msi2 expression by a lentiviral short hairpin RNA interference method. In the control cells, I observed mature myotubes with multiple nuclei and myosin heavy chain (MHC) by immunofluorescence staining at 5 days after differentiation induction. In contrast, Msi2 KD cells showed very few MHC-positive cells in which 1 or 2 nuclei were detected. This result suggests that Msi2 is required for myocyte differentiation and fusion process, which is necessary for mature myotube formation. Interestingly, the protein and mRNA level of Myogenin, an essential MRF that controls MHC expression and terminal differentiation, is not changed by Msi2 KD, suggesting that Msi2 does not regulate the canonical MRF network. Overexpression (OE) of Msi2 successfully rescued the impaired differentiation phenotypes observed in the KD cells, which excluded the possibility of an off-target effect of the Msi2 KD construct I used. Surprisingly, I noticed that Msi2 OE alone generated thicker and longer myotubes 5 days after expression. These results suggest that Msi2 promoted myocyte differentiation and fusion, and is not only necessary but also sufficient for myoblast differentiation.

As a molecular mechanism of myocyte differentiation by Msi2, I speculated that Msi2 regulates mitochondrial function and biogenesis, which are critical factors in myogenesis. Using Mitotracker deep red, an indicator of functional mitochondria, I found the staining level is lower in Msi2 KD cells and higher in Msi2 OE cells compared to the control. I also found that mitochondrial DNA content and mitochondrial respiration function measured by the Seahorse assay were comparable between the control and KD. Since Mitotracker deep red is mitochondrial membrane potential ($\Delta\Psi m$) dependent, these results suggest that Msi2

regulates ΔΨm level without affecting mitochondrial biogenesis. Next, I examined if Msi2 regulates autophagy during myoblast differentiation because ΔΨm level change can both trigger and be regulated by autophagy. First, I found autophagosome marker LC3A-II level correlates with Msi2 expression level during C2C12 differentiation. Msi2 KD reduced both LC3A-II expression and LC3A puncta formation, suggesting that Msi2 KD impairs autophagy during myoblast differentiation. To further test if differentiation defects caused by Msi2 KD are autophagy-dependent, I induced autophagy in control or Msi2 KD cells by using Tat-Beclin1-D11, which is a short peptide that can upregulate autophagy through binding with the autophagy suppressor GAPR-1/GLIPR2. Strikingly, Tat-Beclin1-D11 treatment significantly increased myocyte differentiation and fusion levels in Msi2 KD cells, indicating Msi2 KD phenotype can be rescued by activated autophagy.

To examine the function of Msi2 in skeletal muscle in vivo, I used a Msi2 mutant mouse strain generated by a germline gene-trap strategy. The hind limb muscles of Msi2 mutant mice are also smaller in size and paler in color than wild-type controls (WT). In a treadmill exercise assay, WT mice could run for more than 100 meters compared to only 30 meters on average for Msi2 mutant mice. These results suggest that Msi2 mutant mice have defective skeletal muscle.

In conclusion, this thesis work has established Msi2 as a novel regulator of skeletal muscle development and function by regulating autophagy and provided insights for future studies of Msi2's role in cancer metabolism and myopathies.

（続紙 ２ ）

（論文審査の結果の要旨）

骨格筋を構成する筋細胞の発生及び分化の分子機構については、myogenic regulatory factors (MRFs)と称される一群の転写制御因子の同定や、これらの標的遺伝子の発現における機能や個体発生における役割の解析が主に進められてきた。一方、転写後段階での発現制御が筋分化において果たす役割については不明な点が数多く残されている。申請者は本学位論文において、RNA結合因子Msi2が筋分化に必須であることを明らかにした。培養筋芽細胞からの分化過程において、Msi2の発現阻害によっては、MRFsの発現そのものには影響しないにも関わらず、筋管細胞への分化が抑制されることを見出した。一方、Msi2発現阻害によってオートファジー経路の活性が低下していること、また発現阻害群において細胞透過性ペプチドをもちいてオートファジーを活性化すると、分化阻害が抑圧されることを発見した。また、Msi2を遺伝的に欠損するマウスモデルにおいて、持久走能が野生型と比較して大きく低下していることから、Msi2による筋分化の制御はin vivoにおいても必須の機構であることを明らかにした。

以上述べたように、本論文は筋細胞の分化制御に関して、新たな分子機構の存在とその必要性を明らかにしたものであり、骨格筋の発生制御の更なる理解のみならず、再生障害等を示す筋疾患の治療法開発等にも貢献する可能性を提示するものである。

よって、本論文は博士（薬科学）の学位論文として価値あるものと認める。また、令和４年８月２６日、論文内容とそれに関連した事項について試問を行った結果、合格と認めた。

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