Netrin-4 synthesized in satellite cell-derived myoblasts regulates myotube formation during myogenic differentiation

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Objectives: Satellite cells are resident myogenic stem cells that are indispensable for skeletal muscle regeneration and hypertrophy. In response to injury or physical activity, they start activation, proliferation, and differentiation to form nascent myofibers (myo- tubes). According to previous studies, satellite cells expressed the multi-potent proteins originally found as classical neural axon guidance molecules (ephrins, netrins, semaphorins and slits) during myogenic differentiation (Cho et al., 2021; Siegel et al., 2009; Suzuki et al., 2021). We have focused on the myogenic function of netrin subtypes (especially netrin-1 and -4) and confirmed that netrin-1 would promote fast-type myotube formation (Suzuki et al., 2021). It was also reported that netrin-3 and its cell-membrane receptor neogenin promote myotube formation (Kang et al., 2004). However, the myogenic significances of netrin-4 are still un- clear. Therefore, we assessed the function of netrin-4 on myogenic differentiation in satellite cells-derived myoblasts using netrin-4 knockdown treatment technique and recombinant netrin-4 additional treatment.

Materials and Methods: We performed the knockdown experiments of netrin-4 by specific siRNAs transfection with RNA interfer- ence technique in differentiated satellite cell-derived myoblasts cultures. To assess the myofibertype regulatory function of netrin-4, the mRNA and protein expression of slow- and fast- myosin heavy chain (MyHC) were detected. Immunofluorescence microscopy was also performed to analyze slow/fast MyHC-positive myotube fusion index. To investigate whether netrin-4 promotes myogenic differentiation, the mRNA and protein expression levels of myogenic regulatory factors (MyoD, myogenin Myf5, MRF4) and the ratio of myotube formation (total MyHC-positive myotube fusion index) were measured. Then, we also assessed the effect of ne- trin-4 on myoblasts fusion focusing on Myomaker and Myomixer expression levels. Further, the ratio of myotube formation fol- lowing recombinant additional netrin-4 treatment in differentiated satellite cell-derived myoblasts cultures was measured.

Results and Discussion: Firstly, we evaluate whether netrin-4 affects myofiber-type composition as well as netrin-1 in our previous study (Suzuki et al., 2021). Netrin-4 knockdown experiments revealed that the expression level of fast MyHC was down-regulated and slow MyHC was up-regulated. However, the both of slow and fast MyHC-positive myotube fusion indexes were clearly de- creased. Upon these results, we predicted that netrin-4 would promote myotube formation regardless of myofiber-types. Therefore, next, we measured the total MyHC-positive myotube fusion index following netrin-4 expression knockdown treatment and con- firmed its down-regulation. To verify how netrin-4 regulates myoblasts fusion, the mRNA and protein expression levels of MRFs are measured and revealed that the mRNA and protein expression levels of MyoD were clearly down-regulated. Upon MyoD is recognized as an inducer of Myomaker and Myomixer, we investigated the mRNA and protein expression levels of Myomaker and Myomixer. While only the mRNA expression level of Myomaker was decreased, the inhibition of both the mRNA and the protein expression of Myomixer was observed in netrin-4 knockdown cultures. Finally, to confirm whether netrin-4 induces myotube formation, we also assessed myotube fusion index following recombinant netrin-4 additional experiment and confirmed its up-regula- tion. Thus, netrin-4 synthesized in satellite cells regulates myoblast-myoblast fusion stimulating MyoDmyomixer axis. Taken together with previous findings and this study, the physiological significances of netrins (netrin-1, -3 and -4) in myogenic differentiation are concluded as the induction of myotube formation.

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