Department of Cancer Biology

Division of Genetics
腫瘍抑制分野

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The major interest of this division is in molecular signals that regulate a variety of cellular activities. Our aim is to address and elucidate how dysregulated cellular signals give rise to neoplastic, immune, neural, metabolic, or developmental disorders. Our goal is to understand the molecular bases of tumorigenesis and the development of other intractable diseases as a path toward uncovering therapeutic targets. Currently, we are investigating regulatory mechanisms in protein-tyrosine kinase (PTK)-mediated signaling pathways, their pathophysiological roles and the potential for therapeutic intervention.

1. Activation of the receptor tyrosine kinase MuSK by the cytoplasmic protein Dok-7 in neuromuscular synaptogenesis.

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Protein-tyrosine kinases (PTKs) play crucial roles in a variety of signaling pathways that regulate proliferation, differentiation, motility, and other activities of cells. Therefore, dysregulated PTK signals give rise to a wide range of diseases such as neoplastic disorders. To understand the molecular bases of PTK-mediated signaling pathways, we identified Dok-1 as a common substrate of many PTKs in 1997. Since then, the Dok-family has been expanded to seven members, Dok-1 to Dok-7, which share structural similarities characterized by N-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, followed by Src homology 2 (SH2) target motifs in the C-terminal moiety, suggesting an adaptor function. Indeed, as described below, Dok-1 and Dok-2 recruit p120 rasGAP upon tyrosine phosphorylation to suppress Ras-Erk signaling. However, we found that Dok-7 acts as an essential cytoplasmic activator of the muscle-specific receptor tyrosine kinase (RTK) MuSK in the formation and maintenance of the neuromuscular junction (NMJ), providing a new insight into RTK-mediated signaling. It seems possible that local levels of cytoplasmic activators, like Dok-7, control the activity of RTKs in concert with their extracellular ligands.

The NMJ is a synapse between a motor neuron and skeletal muscle, where the motor nerve terminal is apposed to the endplate (the region of synaptic specialization on the muscle). The contraction of skeletal muscle is controlled by the neurotransmitter acetylcholine (ACh), which is released from the presynaptic motor nerve terminal. To achieve efficient neuromuscular transmission, acetylcholine receptors (AChRs) must be densely clustered on the postsynaptic muscle membrane of the NMJ. Failure of AChR clustering is associated with disorders of neuromuscular transmis-

sion such as congenital myasthenic syndromes (CMS) and myasthenia gravis (MG), which are characterized by fatigable muscle weakness. The formation of NMJs is orchestrated by MuSK and by neural agrin, an extracellular activator of MuSK. However, experimentally when motor nerves are ablated, AChRs form clusters in the correct, central region of muscle during embryogenesis in a MuSK-dependent process known as prepatterning of the receptors. In addition, in vivo overexpression of MuSK causes neuromuscular synapse formation in the absence of agrin, suggesting that muscle-intrinsic, cell-autonomous activation of MuSK may be adequate to trigger presynaptic and postsynaptic differentiation in vivo. However, the mechanisms by which MuSK is activated independently of nerve and agrin had long been unclear.

Because both MuSK and the adaptor-like cytoplasmic protein Dok-7 are localized to the postsynaptic region of NMJs, we examined their interaction and found that Dok-7 is an essential cytoplasmic activator of MuSK. In addition, we found that Dok-7 directly interacts with the cytoplasmic portion of MuSK and activates the RTK, and that neural agrin requires Dok-7 in order to activate MuSK. Indeed, in vivo overexpression of Dok-7 increased MuSK activation and promoted NMJ formation. Conversely, mice lacking Dok-7 formed neither NMJs nor AChR clusters. In addition, we found that postnatal knockdown of *dok-7* gene expression in mice causes structural defects in NMJs and myasthenic pathology, suggesting an essential role for Dok-7 not only in the embryonic formation but also in the postnatal maintenance of NMJs. Furthermore, we found that forced expression of Dok-7 lacking the C-terminal region rescued Dok-7 knockout mice from neonatal lethality caused by the lack of NMJs, indicating restored MuSK activation and NMJ formation. However, these mice showed only marginal activation of MuSK and died by 3 weeks of age apparently due to an abnormally small number and size of NMJs. Therefore, Dok-7's C-terminal region plays a key, but not fully essential, role in MuSK activation and NMJ formation.

Interestingly, mice lacking Lrp4, which forms a complex with MuSK and acts as an essential agrin-binding module, do not show MuSK-dependent AChR prepatterning or NMJ formation. This suggests that Lrp4 is required for MuSK activation under physiological conditions, in contrast to our observation that Dok-7 can activate MuSK in the absence of Lrp4 or its ligand agrin, at least in vitro. Thus, we examined the effects of forced expression of Dok-7 in skeletal muscle on NMJ formation in the absence of Lrp4 and found that it indeed induces MuSK activation in mice lacking Lrp4. However, the activation level of MuSK was significantly lower in the absence of Lrp4. Together, these data indicate that Lrp4 is required for efficient activation of MuSK by Dok-7 in the muscle. Since Lrp4 is also essential for presynaptic differentiation of motor nerve terminals in the embryonic NMJ formation (*Nature* 489:438-442, 2012), this apparent cooperation between Lrp4 and Dok-7 in MuSK activation may be complicated.

The NMJs are cholinergic synapses characterized by ultrastructural specializations, including the presynaptic active zones, the acetylcholine (ACh) release sites of the motor nerve terminal, and the postsynaptic junctional folds of muscle membrane, where ACh receptors (AChRs) cluster in the nearby areas of active zones for efficient neuromuscular transmission. Interestingly, overexpression of Dok-7 in skeletal muscle abnormally activates MuSK, leading to the formation of abnormally large NMJs in mice. However, these mice with abnormally large NMJs show no obvious motor dysfunction. Recently, we have found that Dok-7 overexpression enhances NMJ transmission less markedly than NMJ size. Consistent with this, ultrastructural analyses revealed that the densities of active zones and synaptic vesicles in the presynaptic motor nerve terminals were reduced. In addition, the density and size of postsynaptic junctional folds in the muscle membrane were also reduced. Moreover, terminal Schwann cells (tSCs) exhibits significantly greater penetration of their processes into the synaptic clefts, which connect the pre- and post-synaptic specializations. Together, our findings demonstrate that forced expression of Dok-7 in muscle enhances neuromuscular transmission with significant enlargement and ultrastructural alterations of NMJs, implying increased robustness of neuromuscular transmission. We are investigating Dok-7/MuSK-mediated signaling, including downstream effectors, in regulating formation, maintenance and function of NMJs to develop mechanism-based therapies for NMJ disorders. Recently, we have identified novel downstream genes/proteins critical for NMJ formation and maintenance, including calcium-binding protein 7 (Cabp7) (see below) together with a few cytoplasmic protein kinases.

2. Cabp7 negatively regulates age-related degeneration of NMJs.

Inoue-Yamauchi, A., Eguchi, T., Tezuka, T., Watanabe, Y., Sagara, S., Ozawa, M.[1], and Yamanashi Y.: [1]Core Laboratory for Developing Advanced Animal Models, IMSUT.

As mentioned above, formation and maintenance of NMJs in the central region of the skeletal muscle are governed by MuSK. Interestingly, the transcripts and protein products of AChR subunit and other NMJ-related genes are expressed and accumulated also in the central, synaptic region of myotube in a manner dependent on MuSK. Indeed, we previously reported that midmuscle expression of AChR subunit gene Chrna1 and MuSK transcripts are lost or enhanced in mouse embryos lacking the essential MuSK activator Dok-7 or overexpressing it specifically in

skeletal muscle, respectively. Thus, to identify NMJ-related genes required for the formation and/or maintenance of NMJs, we performed RNA sequencing analysis of the synaptic and extrasynaptic regions of diaphragm muscles in WT mice (3 months old) and found that Cabp7 gene is the most upregulated among significantly upregulated genes in the synaptic region in comparison with the extrasynaptic region. Furthermore, we found that the expression level of the Cabp7 gene is significantly higher in the synaptic region in Dok-7 transgenic (Tg) mice, which overexpress Dok-7 specifically in skeletal muscle, than in wild-type (WT) mice (3 months old). Also, whole-mount in situ hybridization analysis on embryos confirmed that Cabp7 transcripts are specifically expressed in the central region of the diaphragm muscle in Dok-7 Tg and wild-type mice and that the synaptic expression is significantly enhanced in Dok-7 Tg mice compared with that in WT mice. These findings together indicates that Dok-7-MuSK axis regulates expression of Cabp7 gene in skeletal muscle.

To explore the role for Cabp7 in muscle, we generated Cabp7 cKO mice, which lacks Cabp7 protein specifically in skeletal muscle, and found that Cabp7 cKO mice showed a significant reduction in motor function and muscle strength at 12 and 24, but not 3 or 6, months of age in comparison with the controls, as determined by rotarod, forelimb grip, and hindlimb twitch/tetanic force tests. Furthermore, Cabp7 cKO mice showed acceleration of age-related degeneration of NMJs as early as 6 to 12 months of age; namely, axonal swelling, nerve sprouting, denervation and size-reduction of NMJs. Because impaired NMJ function may lead to muscle atrophy and weakness as observed in patients with myasthenia, we investigated if muscle-specific deletion of Cabp7 in mice affects muscle homeostasis and found that tibialis anterior and gastrocnemius muscle masses were significantly reduced in Cabp7 cKO mice at 12 and 24, but not 3 or 6, months of age in comparison with the controls. In addition, the myofiber cross-section area (CSA) of gastrocnemius muscles was significantly reduced in Cabp7 cKO mice at 12 and 24, but not 3, months of age and showed significant shifts in CSA distribution with a higher frequency of small fibers in comparison with the controls. Taken together, these findings indicates that Cabp7 plays a protective role against age-related NMJ degeneration, muscle weakness and atrophy, and motor dysfunction. Indeed, Cabp7 cKO mice showed a lifespan about 8 weeks shorter than the control mice.

In addition, we found that Cabp7 cKO mice showed increased protein expression of p25, a potent activator of Cdk5, in the tibialis anterior muscle at 3 and 12 months of age in comparison with the control mice. Because Cdk5 negatively regulates NMJ formation and maintenance, we generated Adeno-associated virus-based vector (AAV-p25), which expressed p25 in myotubes under the control of the muscle-specific CK8 promoter, and found that AAV-p25 administration induces NMJ degeneration. Moreover, we also generated AAV-CIP, which expressed Cdk5 Inhibitory Peptide (CIP), and found that AAV-CIP administration restores NMJ integrity and muscle strength, and heals muscle atrophy in Cabp7 cKO mice. We are currently investigating how Dok-7-MuSK-Cabp7-p25-Cdk5 axis contributes to NMJ homeostasis and how CIP expression counteracts NMJ degeneration, muscle weakness and atrophy caused by the loss of Cabp7.

3. Agrin's role aside from MuSK activation in the postnatal maintenance of NMJs.

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Although NMJ formation requires agrin under physiological conditions, it is dispensable for NMJ formation experimentally in the absence of the neurotransmitter acetylcholine, which inhibits postsynaptic specialization. Thus, it was hypothesized that MuSK needs agrin together with Lrp4 and Dok-7 to achieve sufficient activation to surmount inhibition by acetylcholine. To test this hypothesis, we examined the effects of forced expression of Dok-7 in skeletal muscle on NMJ formation in the absence of agrin and found that it indeed restores NMJ formation in agrin-deficient embryos. However, these NMJs rapidly disappeared after birth, whereas exogenous Dok-7-mediated MuSK activation was maintained. These findings indicate that the MuSK activator agrin plays another role essential for the postnatal maintenance, but not for embryonic formation, of NMJs. Because pathogenic mutations of agrin in patients with congenital myasthenic syndromes (see below) did not show impaired ability to activate MuSK at least in vitro (*Am. J. Hum. Genet.*, 85:155-167, 2009; *JCI Insight*, 5:e132023, 2020), the novel role of agrin may be relevant to pathogenicity of the mutations. We are investigating molecular mechanisms underlying the agrin-mediated postnatal maintenance of NMJs by utilizing mice expressing various forms of agrin mutants, including those related to congenital myasthenic syndromes (see below).

4. Pathophysiological mechanisms underlying *DOK7* myasthenia.

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As mentioned above, impaired clustering of AChRs could underlie NMJ disorders, be they autoimmune (myasthenia gravis) or genetic (congenital myasthenic syndromes (CMSs)) in origin. Therefore, our findings that Dok-7 activates MuSK to cluster AChRs and to form NMJs suggested *DOK7* as a candidate gene for mutations associated with CMS. Indeed, we demonstrated that biallelic mutations in *DOK7* underlie a major subgroup of CMS with predominantly proximal muscle weakness that did not show tubular aggregates on muscle biopsy but were found to have normal AChR function despite abnormally small and simplified NMJs. We further demonstrated that several mutations, including one associated with the majority of patients with the disease, impaired Dok-7's ability to activate MuSK. This new disease entity is termed "*DOK7* myasthenia."

To investigate pathophysiological mechanisms underlying *DOK7* myasthenia, we established knock-in mice (Dok-7 KI mice) that have a mutation associated with the majority of patients with *DOK7* myasthenia. Dok-7 KI mice showed characteristic features of severe muscle weakness and died by postnatal day 21. Furthermore, they showed abnormally small NMJs lacking postsynaptic folding, a pathological feature seen in patients with *DOK7* myasthenia. Consistent with this, Dok-7 KI mice exhibited decreased MuSK activity in skeletal muscle, indicating that the Dok-7 KI mice develop defects similar to those found in patients with *DOK7* myasthenia, although the mice exhibit a more severe phenotype. In collaboration with Prof. David Beeson's group, we examined NMJ formation, maintenance and functions in the Dok-7 KI mice in the absence or presence of salbutamol, a β2-adrenergic agonist, which is an effective treatment for *DOK7* myasthenia. This study revealed that salbutamol can increase NMJ number and enhance its function together with lifespan in Dok-7 KI mice, suggesting a similar mode of action in patients. We are investigating molecular pathways, especially MuSK-dependent ones, underlying NMJ defects and muscle weakness in Dok-7 KI mice to develop mechanism-based therapeutic approaches against *DOK7* myasthenia.

5. *DOK7* gene therapy that enlarges and regenerates NMJs.

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As mentioned above, *DOK7* myasthenia is associated with impaired NMJ formation due to decreased ability of Dok-7 to activate MuSK in myotubes at least in part. Interestingly, in vivo overexpression of Dok-7 increased MuSK activation and promoted NMJ formation in the correct, central region of the skeletal muscle. Because these genetically manipulated mice did not show obvious defects in motor activity, overexpression of Dok-7 in the skeletal muscle of patients with *DOK7* myasthenia might ameliorate NMJ formation and muscle weakness. To test this possibility, we generated an Adeno-associated virus-based vector (AAV-D7), which strongly expressed human Dok-7 in myotubes and enhanced MuSK activation and AChR cluster formation. Indeed, therapeutic administration of AAV-D7 to Dok-7 KI mice described above resulted in enlargement of NMJs and substantial increases in muscle strength and life span. Furthermore, when applied to model mice of another neuromuscular disorder, autosomal dominant Emery-Dreifuss muscular dystrophy, therapeutic administration of AAV-D7 (*DOK7* gene therapy) likewise resulted in enlargement of NMJs as well as positive effects on motor activity and life span. Interestingly, *DOK7* gene therapy suppressed denervation (nerve detachment) at NMJs, and enhanced motor activity and life span in a mouse model of familial amyotrophic lateral sclerosis (ALS), a progressive motor neurodegenerative disease with severe muscle atrophy. These results suggest potential for *DOK7* gene therapy in age-related decline in motor function, where NMJ denervation appears to play a crucial role similar to that observed in ALS model mice. Indeed, we found that *DOK7* gene therapy significantly enhances motor function and muscle strength together with NMJ reinnervation in aged mice. We are further investigating the effects, including ultrastructural and electrophysiological ones, of AAV-D7 administration in multiple types of muscle weakness.

6. Roles of Dok-1 to Dok-6.

Inoue-Yamauchi, A., Wu, W., Sato, T., Jozawa, H., Kanno, T., Arimura, S.[1], Katayama, K.[2], Imoto, S.[2], Nunès J. A.[3], Andre A.[4], and Yamanashi, Y.: [1]Present affiliation: Department of Medicine Section of Gastroenterology and Hepatology, Baylor College of Medicine. [2]Laboratorye of Sequence Analysis, Human Genome Center, IMSUT. [3]Centre de Recherche en Cancérologie de Marseille, CRCM, Immunity and Cancer Team, Institut Paoli-Calmettes, Inserm, CNRS, Aix Marseille Univ, Marseille. [4]The Laboratory of Molecular Oncology, Institut de recherches cliniques de Montréal.

Dok-family proteins can be classified into three subgroups based on their structural similarities and expression patterns; namely, 1) Dok-1, -2, and -3, which are preferentially expressed in hematopoietic

cells, 2) Dok-4, -5, and -6, which are preferentially expressed in non-hematopoietic cells, and 3) Dok-7, which is preferentially expressed in muscle cells. As mentioned above, Dok-1 and its closest paralog, Dok-2, recruit p120 rasGAP upon tyrosine phosphorylation to suppress Ras-Erk signaling. Although Dok-3 does not bind with p120 rasGAP, it also inhibits Ras-Erk signaling. Consistently, we demonstrated that Dok-1, Dok-2 and Dok-3 are key negative regulators of hematopoietic growth and survival signaling. For example, Dok-1, Dok-2, and Dok-3 cooperatively inhibit macrophage proliferation and *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$ *Dok-3*$^{-/-}$ mice develop histiocytic sarcoma, an aggressive malignancy of macrophages. Also, we found that Dok-1 and Dok-2 negatively regulate intestinal inflammation in the dextran sulfate sodium-induced colitis model, apparently through the induction of IL-17A and IL-22 expression. However, we found that Dok-1/-2 and Dok-3 play distinctive roles in cell fusion and proliferation during osteoclastogenesis and cooperatively protect mice from osteopenia. Additionally, we recently demonstrated that Dok-1/-2 and Dok-3 play distinctive roles in intestinal tumor growth and malignant progression. Interestingly, Dok-3 deficiency in non-tumor cells induces malignant conversion of benign tumors without intensifying mutagenesis in tumors, providing a new insight into the regulation of tumor malignant progression. We are currently investigating molecular mechanisms underlying the Dok-3-mediated suppression of malignant progression of intestinal tumors, which may lead to developing new therapeutic approaches against non-tumor cell-driven malignant progression. In addition, a collaborative investigation with Dr. Veillette and his colleagues revealed the CD200R1-Dok-1/2-Csk inhibitory pathway in phagocytosis. We are further investigating physiological and pathological roles of Dok-1 to Dok-6, including those in T cell memory, macrophage function, tumor malignancy, inflammatory disorders and tissue injury.

7. Omic analyses.

Inoue-Yamauchi, A., Eguchi, T., Jozawa, H., Fan, W., Tokuoka, Y., Yoda, M., Wu, W., Ueta, R., Iemura, S.[1], Natsume, T.[2], Kozuka-Hata, H.[3], Oyama, M.[3], Katayama, K., Imoto, S., and Yamanashi, Y.: [1]Translational Research Center, Fukushima Medical University. [2]National Institute of Advanced Science and Technology, Molecular Profiling Research Center for Drug Discovery. [3]Medical Proteomics Laboratory, IMSUT.

To gain insights into signaling mechanisms underlying a variety of physiological and pathophysiological events, including NMJ formation, muscle atrophy, neurodegeneration, inflammation, tumorigenesis, and tumor metastasis, we have performed proteomic and transcriptomic analyses. We are investigating the roles of candidate proteins and genes that appear to be involved in each of these biological events. For instance, we are conducting transcriptomic and phospho-proteomic analyses related to muscle weakness due to defects of cytoplasmic protein kinases. In addition, we have prepared experimental settings for other omic approaches such as metabolomic analysis.

8. Screening of chemical compound and siRNA libraries.

Inoue-Yamauchi, A., Hwang, J., Eguchi, T., Tsoumpra, M., Ueta, R., Nagatoishi, S., Tsumoto, K., and Yamanashi, Y.

In addition to the omic analyses described above, we performed high throughput screenings of chemical compound and siRNA libraries, aiming to intervene in pathogenic signals or to gain insights into signaling mechanisms underlying NMJ formation. We are investigating in vivo and in vitro effects, including therapeutic ones in mouse models of human diseases, of hit compounds or down- or up-regulation of candidate genes, and continue the ongoing screenings to further collect appropriate hit compounds and candidate genes that may be involved in the regulation of NMJ formation. We are also investigating target proteins, including those in humans, for the hit compounds or protein products of the candidate genes to understand their modes of actions.

Publication

Li Jiaxin, Wang Z, Qin X, Zhong M-C, Tang Z, Qian J, Dou J, Hussell, T, King PD, Nunès JA, Yamanashi Y, Davidson D, Veillette A. CD200R1-CD200 checkpoint inhibits phagocytosis differently from SIRPα-CD47 to suppress tumor growth ***Nat. Comm.*** 16: 5145, 2025