Thesis Summary

Investigation of the role of Clk family proteins in *Xenopus* neural development
(ツメガエル神経形成における Clk ファミリータンパク質の機能解析)

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During early vertebrate development, embryonic cells develop into different cell types along the dorsal-ventral (DV) and anterior-posterior (AP) axes, and this process is mediated by morphogens, including bone morphogenetic protein (BMP), Wnt, fibroblast growth factor (FGF), and retinoic acid (RA). In *Xenopus*, DV patterning is established by neural-inducing factors (Noggin, Chordin, and Follistatin) that emanate from Spemann's organizer and function to induce neural tissue development through inhibition of BMP-mediated epidermal differentiation in dorsal ectodermal cells. AP patterning is regulated by Wnt, FGF, and RA, which promote formation of the mid- and hindbrain as well as the spinal cord through differentiation of the induced neural tissue. During early development, ectodermal cells receive multiple morphogen signals that are important for cell fate decisions. However, the mechanisms of morphogen signal regulation and coordination at the intracellular level are not completely understood.

In this thesis research, I identified a novel function of Cdc2-like kinase (Clk) family proteins, in which they promote neural development of ectodermal cells by regulating morphogen signals. The *clk* family genes encode dual-specificity kinases involved in many biological processes, such as mRNA splicing, cell cycle regulation, and stem cell maintenance. It has been reported that a high level of Clk2 protein contributes to the neurological impairment in Shank3-deficient autism spectrum disorder model mouse. Although the functions of Clk family proteins in adult tissues have recently been discovered, the role of these genes during early vertebrate development is not well understood. In my previous study, I found that Clk2 promotes neural tissue formation in *Xenopus* embryos by regulating morphogen signals, such as BMP and FGF signaling pathways. In addition to Clk2, two other *Xenopus* Clk family proteins, Clk1 and Clk3, have been identified. However, the embryonic function of both Clk1 and Clk3 remains to be elucidated. Therefore, I performed a functional analysis of Clk1 and Clk3 in early vertebrate development using *Xenopus* as a model system.

Firstly, I analyzed the level of identity of *X. tropicalis* Clk1, Clk2, and Clk3 protein

sequences. The protein sequence alignment showed that the kinase domain is well conserved among Clk family proteins, with more than 60% identity. The N-terminal and C-terminal domains of Clk family proteins, on the other hand, are poorly conserved, with less than 35% identity. To determine the function of Clk1 and Clk3 during *Xenopus* embryogenesis, I examined the expression patterns of *clk1* and *clk3* genes. Using RT-PCR analysis, I found that *clk1* and *clk3* are maternally expressed, and their expression is increased, respectively, from the neurula (stage 17/18) and gastrula stage (stage 10.5) in *Xenopus* embryos. Spatial expression analysis of *clk1* and *clk3* using whole-mount *in situ* hybridization showed that both *clk1* and *clk3* are expressed in the dorsal side at the gastrula stage, and their transcripts are specifically distributed within neural tissues along the AP axis at the neurula and tailbud stages. This result indicates that Clk1 and Clk3 may function in the formation of neural tissue during early development. Subsequently, I performed gain-of-function experiments for Clk1 and Clk3 in *Xenopus* embryos. When overexpressed in ectodermal explants, Clk1 and Clk3 induced the expression of early neural marker genes and suppressed the expression of *epidermal keratin*, similar to Clk2. However, among the Clk family proteins, Clk1 had lower neural-inducing activity. Both Clk1 and Clk3 had little effect on the expression of the differentiated neural marker gene *ncam*. Neither Clk1 nor Clk3 overexpression enhanced the expression of the mesodermal marker gene *muscle actin*. These results suggest that Clk family proteins function in promoting neural development without inducing mesodermal formation in *Xenopus* embryos. To determine the roles of endogenous Clk family proteins, I carried out a knockdown experiment using morpholino oligonucleotides (MOs) against *X. tropicalis clk1, clk2,* and *clk3*. Injection of Clk1 MO or Clk2 MO generated a minor phenotypic change, such as a bent axis at the late tailbud stage, in *Xenopus* embryos. In contrast, Clk3 knockdown embryos exhibited severe developmental defects, including reduced head and eye size, a shortened AP axis, and a bent axis. Furthermore, gene expression analysis showed that Clk3 is required for the expression of *ncam* and the neuronal marker gene *n-tubulin*. As expected, there was no reduction in the expression of *muscle actin* in Clk3 MO injected embryos. This indicates that knockdown of Clk3 preferentially affects neural development, rather than mesodermal differentiation, in *Xenopus* embryos. Thus, I concluded that Clk1 and Clk3 promote the neural-fate decision of ectodermal cells, and that Clk3 is an essential factor for neural development during early vertebrate embryogenesis.