氏　　名	森田　仁
学位（専攻分野）	博士（理学）
学 位 記 番 号	総研大甲第1436号
学位授与の日付	平成２３年３月２４日
学位授与の要件	生命科学研究科　基礎生物学専攻 学位規則第６条第１項該当
学 位 論 文 題 目	Analysis of the cellular and molecular mechanisms in vertebrate neural tube formation
論 文 審 査 委 員	主　　査　　教授　　高田　慎治 教授　　上野　直人 教授　　藤森　俊彦 グループディレクター　林　茂生 理化学研究所

Neural tube formation, or neurulation, is one of the most prominent and earliest morphogenetic events and the first organ formation in vertebrate development. This begins with the formation of neural plate, a flat and relatively thick ectodermal tissue on the dorsal side. Subsequently, neural plate bends up as two parallel bulges along anterior-posterior axis, forming neural folds and neural groove. Neural folds finally fuse above the dorsal midline, creating the neural tube, which eventually differentiates into the central nervous system. Defects in these morphogenetic processes result in congenital diseases called neural tube defects (NTDs) in humans.

Neural tube closure proceeds with several kinds of cell shape changes and cellular movements in the neural ectoderm; cells near the midline elongate apicobasally and transform into a wedge-like shape by apical constriction allowing involution of the cells into embryo, and at the same time, cells move toward the dorsal side of the embryo and realign their positions along the anterior-posterior axis by convergent extension movement. These shape changes and cell movements enable the neural ectoderm to form the tertiary tubular structure. Although neural tube formation has been mostly studied with histological analyses, the molecular basis of this process is not fully understood. To better understand this event at molecular and cellular level, the author focused on an adhesion molecule nectin-2 that is strongly expressed in the neural ectoderm in *Xenopus laevis* embryo and investigated its role in the neural tube formation.

Nectin-2 was first identified as a poliovirus receptor-related protein, and it was found to be a cell-cell adhesion molecule in cultured cells and adult mouse organs. Nectin family consists of four members (nectin-1, -2, -3, and -4) in humans and mice with some splicing variants. Nectin proteins have three immunoglobulin (Ig)-like domains in the extracellular region, a single transmembrane region, and a binding motif for afadin, an intracellular molecule that also binds to F-actin. The Ig-like domains of nectin bind to those of another nectin on the adjacent cell surface, facilitating cell-cell adhesion independently of the existence of calcium ions. *In vivo* roles of nectins in developmental processes were studied by generating knockout mice for each *nectin* genes. However, these mice are viable and show subtle phenotypes, such as microphthalmia and male-specific infertility, even though nectins are expressed in almost all tissues in mouse embryos, which was attributed to the redundant functions among nectin family members. In the case of *Xenopus laevis*, the author found this animal also has four nectin genes and among them, *nectin-2* was predominantly expressed in embryonic stages, whereas expression level of other nectin isoforms was significantly low. *Nectin-2* was strongly expressed in the neural ectoderm throughout neurulation, implying that nectin-2 may function in neural tube formation.

The author next analyzed function of nectin-2 by depleting it in early *Xenopus* embryos with injections of a morpholino oligonucleotide (MO) against *nectin-2*. The depletion of nectin-2 from neural ectoderm led to a defective neural tube closure with impaired neural folding. Detailed cellular analyses of this phenotype revealed incomplete apical constriction and loss of apical filamentous actin (F-actin) bundles in nectin-2 MO-injected embryos. Conversely, he injected *nectin-2* mRNA into the ventral side of the embryos, where cells normally do not undergo apical constriction. The ectopic expression of *nectin-2* caused an abnormally pigmented surface ectoderm and some cells in the injected area showed constricted apical surface and enhanced accumulation of apical F-actin, indicating together with the loss of function experiment that nectin-2 may be involved in the apical constriction in neural fold formation.

To identify which domain of nectin-2 is required for its function in apical constriction, the author generated

deletion constructs of *nectin-2* and injected them into the embryos as mRNAs. A mutant nectin-2 that lacks intracellular afadin-binding motif induced ectopic apical constriction and apical F-actin accumulation, whereas another mutant nectin-2 that lacks extracellular Ig-like domains did not, suggesting that the extracellular Ig-like domains are required for apical constriction.

Since nectin-2 protein was previously known to be localized at adherens junctions (AJs) in epithelial cells where another cell adhesion molecule, cadherin is also localized, the author next analyzed the possible interaction of nectin-2 and cadherin for apical constriction. GST pull-down assay with extracellular domains of nectin-2 and three types of cadherins expressed in *Xenopus* embryo revealed that nectin-2 preferentially binds to N-cadherin, a cadherin isoform specifically expressed in neural ectoderm. Then he performed co-expression experiments by injecting mRNAs of *nectin-2* and *N-cadherin* into ventral ectoderm. Low doses of either *nectin-2* or *N-cadherin* alone did not cause any abnormalities, whereas the co-expression of these molecules with the same doses induced the ectopic apical constriction and F-actin accumulation. Subsequently, he carried out a knockdown experiment by injecting MOs against each of these molecules into neural ectoderm to know the involvement of their functional interaction in neural fold formation. Co-injection of both of the MOs attenuated the formation of neural fold more severely than injections of either MO. These results indicate that nectin-2 and N-cadherin cooperatively enhance apical constriction in neural fold formation by the physical interaction via their extracellular domains.

As both nectin-2 and N-cadherin are colocalized at AJs, the author next asked whether their localizations depend on each other. To address this question, he observed the localization of one of nectin-2 or N-cadherin under the depletion of the other. When N-cadherin was knocked down in neural ectoderm, nectin-2 protein was localized at AJs as in the normal embryo, although apical constriction and neural folding were affected. Meanwhile, the reciprocal experiment with nectin-2 knockdown resulted in the significant reduction of apically localized N-cadherin. These results suggest that N-cadherin can be localized at AJs depending on the presence of nectin-2. Finally, he asked whether the intracellular domains of N-cadherin, which is indirectly connected to F-actin, are required for the F-actin accumulation in apical constriction during neural tube formation. Since cadherins are connected with F-actin through binding to α- and β-catenin, he made a mutant N-cadherin construct that lacked intracellular β-catenin-binding site and injected this mRNA into neural ectoderm. Embryos injected with this mutant N-cadherin exhibited the aberrant apical constriction and defective neural fold formation. F-actin staining of these embryos revealed loss of F-actin bundles from the apical side of injected cells, indicating that the F-actin linkage of N-cadherin is essential for apical constriction in neural tube formation.

Based on this and previous studies, the author propose the following model for apical constriction in vertebrate neurulation. At the beginning of neurulation, *nectin-2* and *N-cadherin* start to be strongly expressed in neural ectoderm. Nectin-2 is preferentially localized to the apical AJs by unknown mechanisms. The protein-protein interaction of nectin-2 and N-cadherin through their extracellular domains subsequently recruit N-cadherin dispercedly localized in the plasma membrane to AJs. This relocation of N-cadherin induces apical accumulation of F-actin via the intracellular interaction with N-cadherin. After the accumulation of F-actin, activated non-muscle myosin, an F-actin binding protein, contract F-actin bundles, driving the cells to apical constriction and thus neural tube formation.

In addition to the analysis of nectin-2 and apical constriction machinery in neural tube closure, the author also studied the contribution of non-neural ectoderm to neurulation in the cellular and molecular levels, since previous studies using chick and axolotl showed the requirement of this tissue for normal neural tube closure; in the absence

of the non-neural ectoderm, *i.e.* in an explant containing only neural ectoderm, failed to close the neural tube. To examine whether this is also the case in *Xenopus* neurulation, the author performed explant experiment with or without non-neural ectoderm. The dorsal explants in large size, which contains both neural and non-neural ectoderm, and small size, which consists of neural ectoderm but does not include non-neural tissue, were dissected from the early neurula embryos and cultured until the neural tube of an intact control embryo closes. The neural folds of both explants moved toward the midline as seen in normal neural tube closure. However, sections of the small explant showed that its neural ectoderm formed neural groove and fold but still opened without fusion at the midline to form the tubular structure, whereas those of large explant exhibited closed neural tube, suggesting that the non-neural ectoderm is required for the complete neural tube closure.

It has been known that non-neural ectoderm moves toward the midline, and this movement may contribute to neural tube closure but its cellular and molecular mechanisms remain unclear. Therefore, he next addressed this issue by examining several, previously proposed possibilities. He first tested the involvement of cell division in the neural tube closure using hydroxyurea and aphidicolin (HUA), cell cycle inhibitors. Treatment of embryos with these inhibitors from early neurula stage efficiently suppressed cell division. However, time-lapse observation of neural tube closure revealed that HUA-treated embryos underwent closure of the neural folds in almost the same speed compared with control embryos. Furthermore, sections of the closed neural tubes of control and HUA-treated embryos showed identical structures, suggesting, together with the time-laps observation, that cell division is dispensable for neural tube closure in *Xenopus* embryo. The future works will be focused on other possibilities for the closure mechanisms.

The author hope this study sheds light on the molecular and cellular mechanisms of vertebrate neural tube formation.

博士論文の審査結果の要旨

神経管は将来、中枢神経系を形成する脊椎動物特有の重要な器官で、その形成過程では神経上皮・非神経上皮細胞の特徴的な形態変化や再配置運動が起こる。しかしながら、神経管形成をつかさどる分子・細胞機構にはいまだ不明な点が多い。出願者は神経管形成に関わる細胞動態とその分子・細胞機構を明らかにするために、神経上皮と非神経上皮それぞれの運動について、アフリカツメガエル胚を用いて細胞・分子レベルでの解析を行った。出願者はまず、初期発生、器官形成における機能がほとんど知られていない細胞接着分子ネクチンが神経管領域に強く発現していることを見出した。ツメガエル胚でネクチンの機能を阻害したところ、神経上皮のアクチンの集積や神経上皮細胞の一部に見られる特徴的な細胞形態変化である頂端収縮が阻害され神経管閉鎖の著しい遅れが生じたことから、ネクチンがアクチン集積を介した頂端収縮に必要なことが示唆された。次にネクチン mRNA を本来頂端収縮が見られない胚腹側に過剰発現させたところ、異所的なアクチン集積と頂端収縮が引き起こされたことによって、阻害実験から予想されたネクチンの機能が裏付けられた。続いてドメイン欠失型ネクチンを用いた過剰発現実験により、ネクチンの細胞外部位が頂端収縮に必須であることを示した。また、ネクチンとの共局在が知られている接着分子・カドヘリンに着目し、両者の機能的相互作用について検証した。ネクチンと神経板で特異的に発現するN-カドヘリンとの結合実験などから、ネクチンとN-カドヘリンが相互作用し協調的に頂端収縮を制御することを明らかにした。さらにN-カドヘリンの頂端側への集積はネクチン依存的であったことから、ネクチンはN-カドヘリンの結合によってアクチンを細胞頂端に集積させることで頂端収縮を引き起こすという脊椎動物の神経管形成過程における分子・細胞機構の一端を明らかにした。

次に、非神経上皮細胞の移動の原動力について解析を行った。まず神経管閉鎖における非神経上皮の必要性について背側外植体を用いて検証し、非神経上皮の除去により神経管が完全に閉鎖しないことを見出し、正常な神経管閉鎖に非神経上皮が必要なことを示した。また、非神経上皮は細胞増殖を介して神経管閉鎖に寄与するという過去の報告に基づき、非神経上皮の移動への細胞分裂の寄与を阻害剤処理によって検証し、神経管閉鎖には細胞分裂は必要でないことを明らかにした。

以上のように、本研究は神経上皮と非神経上皮での細胞動態をそれぞれ解析することで、神経管閉鎖という脊椎動物の発生における重要な形態形成運動の分子レベル、細胞レベルでの理解を進めた。とくに頂端収縮の機構の解明は、他生物種や他の器官形成における同様な細胞形態変化を伴う形態形成運動の理解に貢献しうる成果であると評価され、審議の結果、本研究成果は学位授与に相応しいと判断した。