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学位論文名	**細胞接着と細胞間シグナルによる下垂体前葉の前駆細胞維持機構**
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論文内容の要旨

1　研究目的

Existence of stem/progenitor cell in adult tissue and its maintenance throughout livelihood is important matter that is taking lots of interests from researchers. Tissue specific stem cells have been identified in many organs, not only in embryonic stage but also in adult stage and play important roles in normal tissue homeostasis and to regenerate the specific cells after lesion. The anterior pituitary is one of the major regulator of mammalian physiology, in terms of secreting endocrine factors that control many physiological processes. However, how they are maintained properly in adult stage is still uncertain. Among multiple regulatory mechanisms that generally governs the stem/progenitor cell, Notch signaling is one of cell-to-cell signaling pathways, which regulates several genes that are associated with stem cell differentiation and proliferation in several organs. Recently, our group shown that its involvement in anterior pituitary stem/progenitor cell proliferation. E-cadherin is one of the primary adhesion molecule that is restricted to stem/progenitor cell of anterior pituitary gland all throughout embryonic stage to adult stage. However, E-cadherin and Notch signaling relation in adult anterior pituitary gland have not been fully studied. Sox2 is a transcription factor that is known as stem/progenitor cell marker in many tissues, which was also found in anterior pituitary gland. It possess an ability to differentiate into all types of hormone producing cell of the gland. Even though relation of Sox2 and Notch signaling been studied, histological relation of two molecule have not been succeeded. The aim of my study was to identify the relation between E-cadherin expression and Notch signaling molecules and to clarify our hypothesis that possible involvement of E-cadherin on Notch signaling in the anterior pituitary gland of adult rats. I also aimed to clarify the localization of Notch signaling and SOX2, further to elucidate role of Notch signaling in SOX2 expression.

2　研究方法

Experiment I was carried out to find the role of E-cadherin in Notch signaling. Using male

adult Wistar rats, immunohistochemistry was performed to clarify the histological characterization of molecules in subject. Primary cell culture was utilized to find E-cadherininhibition effect on Notch signaling, which followed by immunocytochemistry. The Aim of the Experiment II was to elucidate possible role of Notch signaling on SOX2 expression. Immunohistochemistry and in situ hybridization were carried out on embryonic stage and adult pituitary gland. Primary cell culture was performed using anterior pituitary cells, which were treated with Notch signaling inhibitor, DAPT, to demonstrate the Notch signaling effect on Sox2-positive cell expression.

3　研究成果

Experiment I, identified that two of Notch signaling receptors, Notch1 and Notch2, are expressed within E-cadherin-positive cell clusters of anterior pituitary cells and in marginal cell layer cells, with the exception of Notch1 expressed in E-cadherin-negative endothelial cells of vasculature. Notch signaling ligand Jagged1 as well as the Notch target HES1 were restricted to E-cadherin-positive cells in both marginal layer cells and in the anterior pituitary. Co-localization of Notch2 and Jagged1 were observed by immunohistochemistry. Inhibition of E-cadherin adhesion resulted in the distinct decrease in Notch signaling, which was carried out by using primary cell culture and immunocytochemistry for Notch signaling target Hes1.

In Experiment II, in situ hybridization and immunohistochemistry clarified that Notch2 and SOX2 are co-expressed in the same cells in both embryonic stage and the adult stage. Notch target Hes1 was expressed in SOX2-positive cells in the marginal layer cells and in main part of pituitary gland, except for the cells that are expressed in the vasculature. Inhibition of Notch signaling on primary cell culture using Notch signaling inhibitor, DAPT, resulted in number of $SOX2^{+}$ cells.

4　考察

In Experiment I, Notch2 precisely coexisted with E-cadherin and Notch2-positive cells appeared to be a subpopulation of E-cadherin–positive cells. In contrast, Notch1 was expressed in E-cadherin–positive and –negative cells. In rat pituitary gland, Notch2 seemed to be closely associated with FS cells and marginal layer cell function, whereas Notch1 had roles in these and other cells. I could show that Notch1-positive cells not accompanied by E-cadherin belonged to the vasculature, suggesting that Notch1 has a role in vascularization in the anterior pituitary gland. Notch signaling ligand Jagged1 was expressed in E-cadherin–positive cells in the marginal cell layer and in the main part of the anterior pituitary gland. Whereas Notch receptors are known to share ligands, the combination of Notch2 and Jagged1 might be specific to the functions of FS cells and marginal layer cells. A unique finding of this study is that the Notch signaling receptor Notch2 and ligand Jagged1 are always co-expressed in the

same cells. Usually, Notch ligand and receptor are separately expressed in neighbouring cells. Studies showed that co-expression of ligand and receptor in the same cell results in the nullification of Notch signaling within the cell. These results indicate that the co-expression of receptor and ligand does not cause substantial inhibition in the anterior pituitary. Instead, Notch signaling is accomplished by homophilic cell adhesion. Notch target HES1 was expressed in E-cadherin–positive cells in the marginal cell layer and in the main part of the anterior lobe, which suggests that Notch signaling is active within these cell clusters. Our group previously reported that in the pituitary gland, E-cadherin expression is restricted to non–hormone-producing cells. Expression of Notch and its ligands might be restricted to specific phenotypes as stem/progenitor cells. In contrast, HES1 signals outside E-cadherin–positive cells are considered Notch1-associated reactions in the vasculature without ligand expression, which suggest that Notch signalling in the vasculature is activated through non-canonical pathway. Further, by inhibiting E-cadherin with a specific antibody (DECMA-1) in primary culture, we examined whether E-cadherin–mediated adhesion is necessary for Notch signaling in anterior pituitary cells. The proportion of HES1-expressing cells to total Notch2-positive cells was more than 50% lower in the E-cadherin–inhibited culture, suggesting E-cadherin–mediated close immediate contact between cells is necessary for the activation of Notch signaling in the anterior pituitary gland.

In Experiment II, combining *in situ* hybridization and immunohistochemistry or utilized double immunohistochemistry, at embryonic day 14.5, showed that *Notch2* is observed in most cells of the prospective *pars intermedia* and *pars distalis*, but not in the prospective *pars tuberalis*. Moreover, Notch2 expression was mostly accompanied by nuclear SOX2. In the adult pituitary gland, Notch2 molecule was observed on the cells in marginal cell layer and specific cell aggregates in the main part of anterior lobe, which co-expressed with SOX2. Furthermore, SOX2 was always accompanied by Notch signaling target gene HES1, except for HES1 expression that was considered in the endothelial cells, as mentioned above. This suggests that SOX2 expression is maintained specifically in Notch-positive cells in the anterior pituitary glands throughout embryonic and adult stages. *In vitro*, when Notch signaling inhibitor DAPT was added to the culture medium, the number of SOX2-expressing cells within Notch2-expressing cells was significantly lower than that of control. Taken together with our previous report, which showed relation between Notch signaling and cell proliferation, it may be considered that Notch signaling is associated with both cell proliferation and maintenance of stemness of progenitor cells in the anterior pituitary gland.

5　結論

This study suggested a hypothesis that stem/ progenitor cells are maintained in adult rat pituitary through homophilic cell adhesion and local cell signaling among them, and Notch

signaling plays important roles in regulating cell proliferation and maintaining stemness. Specific expression of E-cadherin may be responsible to construct the homophilic cell adhesion.

A unique point of this model is that each stem/ progenitor cell behaves as both signal-sending and receiving cells reciprocally. Results of these studies help to lay the groundwork for future research on anterior pituitary gland stem/progenitor cell maintenance, function, development, and disease.

論文審査の結果の要旨

要約； 申請者は腺性下垂体の幹細胞維持機構に焦点を置き、そのシグナル伝達機構に関わる一連の分子を明らかにした。下垂体前葉の幹細胞と考えられる細胞群は E-カドヘリン陽性であり、その中でも Notch2 とそのリガンドである Jagged1 を共発現している細胞が幹細胞であることが示唆された。それらの細胞では、Notch シグナルの標的遺伝子であり、幹細胞性に関与する転写因子である Hes1 が発現しており、E-カドヘリンを抗体で阻害すると Hes1 の発現が低下することから、これらのシグナル伝達機構が幹細胞性の維持に関与することが示唆された。さらに、幹細胞のマーカーとされる SOX2 が Hes1 と共局在しており、Notch 系の阻害剤により SOX2 の発現が減弱することからも、Notch 系が幹細胞維持に重要であることが示唆された。また、これらの幹細胞に T-カドヘリンが共発現していることも追加で示された。

評価； 申請者は、まだ詳細が不明な下垂体前葉における幹細胞維持の機構の一端を明らかにした。これは、他にも幾つかのシグナル系が報告されてはいるが、幹細胞を利用した下垂体性疾患の治療や下垂体腫瘍の発生機構解明などにも関連していく可能性があり、新規性も十分である。

しかし折角初代細胞培養系を用い阻害剤の実験を行っているのに、Notch シグナルの活性化の指標である NICD の解析等、実際のシグナル活性化の途中の検討がなく、ノックダウン実験は不調であったということで、細胞組織学的実験をサポートするデータがもっとあれば、より良い仕事になったと思われる。また、審査員からの指摘があったが、HES1, SOX2 の発現により幹細胞性を評価しているが、幹細胞性のより詳細な検討、また分化の程度など他の生理的な指標も検討するとさらに良かったと考える。

全体的には良くこなされた仕事で、論文も良く書かれており、誤りも非常に少なかった。また、学位論文の内容より英文雑誌に２報アクセプトされており、高い評価をつけ得ると考える。

問題点等； E-カドヘリンの阻害抗体を用いた実験で細胞接着が疎になった可能性が示唆されていたが、細胞凝集などの詳細な評価のないことが指摘された。

また、Jagged1 と Notch が共発現していると cis-inhibition の可能性があるところを、HES1 が発現していることより活性化されていると結論付けていたが、この分子機構の検討が不十分である。Notch 系が構成的に活性化されているという考察も裏付けとなる NICD の検討等が欲しかった。

さらに幹細胞性の指標として SOX2 を検討していたが、他の Oct4 や CD44 の発現などの検討などの幹細胞性のさらなる検討、Notch の阻害により分化マーカーなどは変化するかなどの検討もあると

良かった。また、幹細胞の self-renewal や分化能に関する記載、さらに生理学的病態的な記載の少ないことも指摘された。

少なかったが、誤字や図の誤りなどは、申請者によって訂正が行われた。

合否；本論文は、医学部博士課程として十分なデータ量とそれを裏打ちする仕事量を持ち、２報の英文論文もアクセプトされており、審査員一同、本学の学位論文の基準を満たすと判断した。

最終試験の結果の要旨

申請者による発表は、データの流れが明瞭で理解しやすかった。研究目的から実験方法、結果への進行も自然で納得のいくものであった。これは、結果を二部構成にしたのが良かったものと思われる。

審査員からの質問に対しても真摯に受け答えし、現段階での限界点や課題も理解しており、一般的な質問に対しても、勉強が伺える返答をしていた。

以上より、審査員は全員一致で申請者が医学博士号を受けるに値すると判断し、最終試験を合格とした。