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学位授与の日付	平成３１年３月３１日
論　文　名	Expression and localization of ephrin-B1, EphB2 and EphB4 in the mouse testis and epididymis in the adult and during the postnatal development （成体と生後発達期のマウスの精巣と精巣上体における ephrin-B1，EphB2，EphB4 の発現と局在について）
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論文要旨

Introduction

The testis and epididymis are composed of a series of a tubule/ductule/duct system lined with tissue-specific epithelia. The intratesticular excurrent duct system consists of straight tubules and the rete testis. Seminiferous tubules generally form loops and both ends are connected to straight tubules which empty into the rete testis. The efferent ductules, the initial part of the extratesticular excurrent duct system, connect the rete testis with the ductus epididymis. The epididymis is grossly divided into three parts: the caput, corpus, and cauda epididymis but can be divided into more segments (I-V) histologically and histochemically. The seminiferous/straight tubules and rete testis originate from the testicular cords, whereas the ductus epididymis originates from the mesonephric duct and the efferent ductules from mesonephric tubules. Therefore, two developmental boundaries are assigned to the junction at the proximal and distal end of the efferent ductules in the genital tubule/ductule/duct system, and these boundaries are histologically maintained in the epithelia of adult tissues. However, the mechanisms underlying the maintenance of these boundaries accompanied by the morphological transition of the epithelia are not completely clear.

Eph receptors and ephrin ligands serve as a cell–cell communication system with a variety of roles in normal development, physiology, and disease pathogenesis. In mammals, Eph receptors are divided into EphA (A1–A8 and A10) and EphB (B1–B4 and B6) classes and members of these two receptor classes promiscuously bind to ligands of the ephrin-A (A1–A5) and ephrin-B (B1–B3) classes, respectively. EphB/ephrin-B signaling is implicated in maintaining epithelial integrity, homeostasis and boundary formation in variety of epithelia. Thus, EphB receptors and ephrin-B ligands might play a role also in the epithelial boundary formation of the male genital system; however, their expression in the testis and epididymis has not yet been examined. Therefore, the author investigated the expression and localization of EphB receptors and ephrin-B ligands in the mouse testis and epididymis, especially to determine whether their expression boundaries exist in the series of the tubule/ductule/duct system in the adult and when their expression boundaries are formed/completed during the postnatal development if existed.

Chapter 1: Expression and localization of ephrin-B1, EphB2 and EphB4 in the adult mouse testis and epididymis

Section 1: Ephrin-B and EphB expression in the adult mouse testis and epididymis

Transcripts for all mammalian ephrin-B and EphB molecules were detected in both the testis and epididymis. EphB2 and EphB4 proteins were detected by western blot with immunoprecipitated samples in both the testis and epididymis. Moreover, EphB2 in the testis and EphB4 in the testis and epididymis were tyrosine-phosphorylated, suggesting that these receptors are activated in the adult testis and/or epididymis *in vivo*.

Section 2: Ephrin-B1, EphB2 and EphB4 localization in the adult mouse testis and epididymis

Ephrin-B1 immunoreactivity was faint or almost negative in the epithelial cells lining of straight tubules, strong in the rete testis, and weak in the efferent ductules. Ephrin-B1 was also expressed in epithelial cells of all segments of the ductus epididymis. In particular, ephrin-B1 was strongly expressed in segments I, IV, and V of the ductus epididymis. In contrast, EphB2 immunoreactivity was detected in epithelial cells lining the rete testis weakly and efferent ductules strongly, but not in those lining the straight tubules. EphB4 also expressed strongly in epithelial cells lining of straight tubules and efferent ductules and weakly in rete testis. EphB2 and EphB4 immunoreactivity was faint or almost negative in those of the epididymis. Summarizing the above, the ephrin-B- and EphB-predominant expression compartments appear alternately along the excurrent system. Thus, the compartmentalization based on the ephrin-B and EphB expression patterns corresponds to the histological as well as the developmental compartments in the excurrent duct system. These expression patterns suggest that strong EphB/ephrin-B signaling likely arises at every epithelial junction along the excurrent system of the testis and epididymis. Therefore, the author proposes that the interplay between EphB and ephrin-B may

regulate/maintain epithelial boundaries along the excurrent system of the testis and epididymis.

Moreover in the testis, peritubular myoid cells expressed ephrin-B1 and EphB4; Leydig cells expressed ephrin-B1 strongly and EphB4 moderately; spermatogonia weakly expressed ephrin-B1 and EphB4; elongated spermatids attached to Sertoli cells expressed EphB2. In the epididymis, smooth muscle cells expressed ephrin-B1 and EphB4; fibroblasts in the interstitial tissue expressed ephrin-B1, EphB2 and EphB4. These findings suggest that EphB4/ephrin-B1 signaling likely arises in spermatogonia, in Leydig cells, between peritubular myoid cells and Leydig cells, and in fibroblasts and smooth muscle cells surrounding ductus epididymis. This may partly support by the findings: EphB4 was tyrosine-phosphorylated in the testis and epididymis. Furthermore EphB2 was expressed strongly in immature spermatozoa located in lumen of proximal parts of the excurrent duct system but EphB2 immunoreactivity in spermatozoa gradually decreased from segment II to segment V of epididymis, in which spermatozoa were almost EphB2-negative, indicating that EphB2 is unlikely required by mature spermatozoa. Thus EphB2 expression can be a new negative indicator for the maturation of spermatozoa.

Chapter 2 Expression and localization of ephrin-B1, EphB2 and EphB4 in the mouse testis during the postnatal development

During the postnatal development (1 day to 8 weeks of age) of the testis, ephrin-B1, EphB2 and EphB4 were expressed at all postnatal ages examined but their relative expression levels were decreased with age up to 4 weeks, and thereafter the levels were almost unchanged until 8 weeks. These indicate that the expression levels of ephrin-B1, EphB2 and EphB4 are close to those in the adult until 4 weeks of age.

Ephrin-B1, EphB2 and EphB4 were faintly or weakly expressed uniformly in epithelia of the intratesticular excurrent duct system at 1 day and 1 week of age. Ephrin-B1 and EphB2/EphB4 expression compartment appeared in the epithelia from 2 weeks of age: ephrin-B1 immunoreactivity was faint in the straight tubules, strong in the rete testis, and weak in the efferent ductules whereas EphB2/EphB4 immunoreactivity was weak in the rete testis and strong in the efferent ductules; in the straight tubules EphB2 immunoreactivity was faint but EphB4 was strong. These findings indicate that the ephrin-B1- and EphB2/EphB4-predominant expression compartments appeared in the intratesticular excurrent duct system until 2 weeks of age. Therefore the intratesticular excurrent duct system is completed far earlier than the time when spermiation starts in terms of ephrin-B1 and EphB2/EphB4 expressions. Moreover, ephrin-B1 and EphB4 immunoreactivities were almost negative in germ cells/gonocytes at 1 day but turned to be positive in spermatogonia from 1 week of age. The relative ratio of stromata to seminiferous tubules was rapidly decreased from 1 day towards 3-4 weeks of age. Stromal cells expressed ephrin-B1, EphB2 and EphB4: fibroblasts expressed ephrin-B1, EphB2 and EphB4 substantially until 2-3 weeks of age and faintly or not thereafter; peritubular myoid cells and Leydig cells expressed ephrin-B1 and EphB4 in all ages during the postnatal development; in Leydig cells

ephrin-B1 immunoreactivity increased until 4 weeks of age while EphB4 immunoreactivity remained unchanged during the postnatal development.

Chapter 3 Expression and localization of ephrin-B1, EphB2 and EphB4 in the mouse epididymis during the postnatal development

During the postnatal development (1 day to 8 weeks of age) of the epididymis, relative expression levels of ephrin-B1 and EphB2 were decreased gradually with ages until 4 weeks, and thereafter they were almost similar up to 8 weeks. Relative EphB4 expression levels did not differ up to 2 weeks of age, then decreased sharply toward 4 weeks, and were almost unchanged up to 8 weeks. These indicate that the expression levels of ephrin-B1, EphB2 and EphB4 become close to those in the adult until 4 weeks of age.

Ephrin-B1 immunoreactivity was uniform in epithelia of the efferent ductules and the ductus epididymis until 2 weeks of age while it started to differ among segments of the ductus epididymis at 3 weeks and was close to the adult pattern at 4 weeks of age: ephrin-B1 was strongly expressed in epithelia of segments I, IV, and V. In contrast, EphB2 and EphB4 immunoreactivity was detected in epithelia of efferent ductules of all postnatal ages. EphB2 immunoreactivity was faint or not in epithelia of the ductus epididymis of all postnatal ages while EphB4 immunoreactivity was weak in epithelia of the ductus epididymis until 2 weeks and then abruptly disappeared in principal cells but was still faint in basal cells at 3 weeks and thereafter. These findings indicate that ephrin-B1 and EphB2/EphB4 expression compartment formed in the excurrent duct system of the epididymis until 3 weeks of age. Therefore the excurrent duct system in the epididymis may be completed until 3 weeks of age in terms of ephrin-B1 and EphB2/EphB4 expressions. Moreover, the relative ratio of stromata to epithelia in the epididymis was rapidly decreased from 1 day towards 3-4 weeks of age: ephrin-B1, EphB2 and EphB4 were uniformly expressed in stromal cells in early postnatal ages and their expressions converged to stromal cells definitely differentiated and localized in the epididymis until 2 weeks of age.

Conclusions

1. The ephrin-B1- and EphB2/EphB4-predominant expression compartments appear alternately along the excurrent duct system, which is lined with the tubule/ductule/duct-specific epithelia. This compartmentalization corresponds to the histological as well as the developmental compartments.
2. EphB2 is expressed in immature spermatozoa but not in mature spermatozoa located in the ductus epididymis of the cauda epididymis. Therefore EphB2 expression can be a new negative indicator for the maturation of spermatozoa.
3. The complementary expression patterns of ephrin-B1 and EphB2/EphB4 are formed until 2 weeks of age in the intratesticular excurrent duct system and until 3 weeks of age in the epididymal excurrent duct system during the postnatal development.

4. Ephrin-B1 and EphB4 are expressed in spermatogonia but not in gonocytes.
5. Epithelia of the ductus epididymis express ephrin-B1 in all ages during the postnatal development and EphB4 in early postnatal ages up to 3 weeks.

審査結果の要旨

精巣は精子形成とアンドロジェン合成を担い，精巣上体は精子成熟の場である。精巣と精巣上体の実質は一続きの細管系で構成される。この細管系は，精巣では精子形成を担う曲精細管とこれに続く直精細管および精巣網から，精巣上体では屈曲する 1 本の精巣上体管から成り，精巣網と精巣上体管は数本の精巣輸出管で結ばれる。直精細管以降の一連の細管系は精子流出管系と呼ばれ，それぞれの区画で異なる形状・性状を示す上皮細胞で形成されている。また，この細管系は発生学的に生殖索，中腎細管，中腎管から分化し，生殖索から精細管と精巣網, 中腎細管から精巣輸出管, 中腎管から精巣上体管が形成される。精子流出管系の形成過程で上皮細胞が移動して混じり合うことはなく，発生期の流出管系の区画は成体でも維持されているが，この維持機構はよく分っていない。

一方，本研究の対象分子 Eph 受容体キナーゼと ephrin リガンドは膜タンパクで，各々A, B サブクラスに大別され（EphA1~A8, A10; EphB1~B4, B6; ephrin-A1~A5; ephrin-B1~B3），同じサブクラスであれば結合する。Eph 発現細胞と ephrin 発現細胞が接触すると，両細胞にシグナルが発生し，細胞の接着や遊走を制御する。最近，胃腸の消化管粘膜，乳腺において ephrin-B1, EphB2 と EphB4 が上皮の恒常性や境界形成の維持に働くことが報告された。これまでに，精巣と精巣上体において Eph と ephrin の発現・局在と働きを調べた報告は認められず，詳細は不明である。そこで，本研究では成体と生後発達期のマウスの精巣と精巣上体を対象に，特に精子流出管系の上皮に着目し，ephrin-B1，EphB2 と EphB4 の発現と局在を検討した。

第1章では, 成体マウス（8〜9週齢）の精巣と精巣上体を材料に RT-PCR, ウエスタンブロット，免疫染色で発現と局在を検討した。その結果, (1) ephrin-B1，EphB2，EphB4 の mRNA は精巣と精巣上体に発現し，精巣に発現する EphB2 と精巣上体に発現する EphB2 と EphB4 タンパクはリン酸化し，活性化していることが明らかになった。流出管の上皮において，(2) ephrin-B1 は精巣網と精巣上体管，EphB2 は精巣輸出管，EphB4 は直精細管と精巣輸出管に強く発現することを見出した。従って，ephrin-B1 を強く発現する区画（精巣網，精巣上体管：特に Segment I, IV, V）と EphB2/EphB4 を強く発現する区画（直精細管，精巣輸出管）が精子流出管の中で交互に出現することが判明し，ephrin-B1 と EphB2/EphB4 は区画化の維

持に働く可能性があることが示唆された。流出管以外では，(3)精巣において，精祖細胞，ライディッヒ細胞，精細管周囲の筋様細胞が ephrin-B1 と EphB4 を，伸長型精子細胞と精子が EphB2 を発現していること，(4)精巣上体において，近位・中位側の精巣上体管の管腔内の精子は EphB2 を発現し，遠位側ではこの発現は消失していることを見出した。この点から，EphB2 の発現消失は精子の成熟指標になることが示唆された。

第 2 章では，生後発達期(1 日～8 週齢)の精巣を材料に RT-PCR と免疫染色で発現と局在を検討した。その結果，ephrin-B1，EphB2，EphB4 の mRNA の発現レベルは，生後 1 日～4 週齢にかけて減少し，その後一定のレベルで発現が持続していることが判明した。精子流出管の上皮において，ephrin-B1，EphB2，EphB4 はいずれも，生後 1 週間までの発現レベルは低く，2 週以降に ephrin-B1 を強く発現する区画(精巣網)と EphB2/EphB4 を強く発現する区画(直精細管，精巣輸出管)が出現し，成体に類似した発現パターンが認められた。曲精細管からの精子離脱は 5 週齢前後で起こるため，ephrin-B1 と EphB2/EphB4 発現による精巣内流出管の区画化はかなり早期に起こることが判明した。曲精細管において，1 日齢の生殖細胞に ephrin-B1，EphB4 発現は認められず，1 週齢以降に明確な発現が認められた。従って，始原生殖細胞から精祖細胞への分化に伴い ephrin-B1/EphB4 発現が出現することが示唆された。

第 3 章では，生後発達期(1 日～8 週齢)の精巣上体を材料に RT-PCR と免疫染色で発現と局在を検討した。その結果，ephrin-B1 と EphB2 の mRNA の発現レベルは，生後 1 日～4 週齢にかけては減少し，その後一定のレベルで発現が持続していること，EphB4 の発現レベルは，生後 2 週齢まではほぼ一定で高く，3 週齢で急激に減少し，4 週齢以降一定のレベルで発現が持続していることが判明した。精子流出管の上皮において，ephrin-B1 は生後 2 週齢までは発現レベルは低く，3～4 週齢にかけて ephrin-B1 を強く発現する区画(精巣上体管の Segment I, IV, V)が明瞭になった。EphB2 は 3 週齢以降で強く発現する区画(精巣上体の精巣輸出管)が明瞭になった。一方，EphB4 は生後 2 週齢までは精巣輸出管と精巣上体管の両上皮に同じレベルで発現していたが，3 週齢以降は精巣上体管の発現はほぼ消失し成体の発現パターンを示すようになった。5 週齢で精巣上体管に精子の貯留が見られるため，ephrin-B1 と EphB2/EphB4 発現による精子流出管の区画化はかなり早期(3 週齢)に起こることが判明した。

これらの成果から，ephrin-B1，EphB2，EphB4 は流出管系の区画化に関与する可能性があること，EphB2 の発現消失は精子の成熟指標になること，ephrin-B1/EphB4 は始原生殖細胞から精祖細胞への分化指標になることが示唆された。本研究は，精子発生・成熟と精子流出管系の分化・維持に関する新たな情報を提供していることから，基礎獣医学ならびに基礎医学の発展・展開に大きく貢献するものと考えられる。従って，本論文の審査ならびに最終試験の結果と併せて，博士(獣医学)の学位を授与することを適当と認める。