생후 발달과정 동안 숫 흰쥐의 Efferent Ductules과 부정소에서 Cytochrome P450 Aromatase (*Cyp19*) 발현 양상 분석

김주영* · 서희정* · 김옥순* · 김병준** · 이성규* · 백행운* · 이기호*

을지대학교 생화학-분자생물학교실 및 의과학연구소*, 내과학교실**

Analysis of Differential Expression of Cytochrome P450 Aromatase (*Cyp19*) in The Efferent Ductules and The Epididymis of Male Rats During Postnatal Development

Ju-Young Kim*, Hee-Jung Seo*, Ok-Soon Kim*, Byung-Joon Kim**, Seong-Kyu Lee*, Haing-Woon Baik* and Ki-Ho Lee*

Departments of Biochemistry and Molecular Biology and Medical Sciences Research Institute* and Internal Medicine**, Eulji University School of Medicine

요 약

본 연구는 생후 발달과정에서 숫 흰쥐의 생식기인 Efferent ductules (EDs)과 부정소에서 cytochrome P450 aromatase (*Cyp19*) 유전자의 발현과 연령별 발현 양상을 알아보는데 목적이 있다. 조직으로부터 추출된 total RNA는 역 전사 반응을 통해 cDNA로 바뀌어진 후 real-time PCR 기법을 사용하여 부위별 그리고 연령별로 *Cyp19* 유전자 발현을 알아보았다. *Cyp19* 유전자의 발현은 EDs에서 90일령을 제외한 7일, 14일, 그리고 30일령 등 성숙기 이전의 모든 연령에서 나타났으며, 부정소에서는 7일령을 제외한 모든 연령에서 보여졌다. 특히 부정소에서 *Cyp19*의 발현은 부정소의 각 부위별로 특이한 양상을 보였다. 본 연구 결과를 통해 남성 생식기의 EDs와 부정소의 각 부분에서 *Cyp19* 유전자 발현은 연령과 부위에 따라 차별적으로 조절되는 것을 알 수 있었다.

(**Key words :** Efferent ductules, Epididymis, Cytochrome P450 Aromatase, mRNA expression)

Ⅰ. INTRODUCTION

The male reproductive tract is composed of the testis and the excurrent duct. Spermatozoa produced from the testis become mature and acquire fertilizing capacity throughout traveling the excurrent duct, the efferent ductules (EDs) and the epididymis. The EDs are tubular structure that has a lumen inside surrounded by a single layer of epithelia (Lee et al, 2000). The EDs have a number of functions, including secretion of ions and protein, absorption of testi-

Corresponding author : Ki-Ho Lee, Department of Biochemistry and Molecular Biology and Medical Sciences Research Institute, College of Medicine, Eulji University, 143-5 Yongdoo-dong, Joong-goo, Daejeon, 301-746, Korea.
Tel : 82-42-259-1643, Fax : 82-42-259-1649, E-mail : kiholee@eulji.ac.kr

cular protein, and reabsorption of the testicular fluid to efficiently increase concentration of luminal sperm (Ilio and Hess, 1994). In male reproductive tract, sperm maturation and storage occur in the epididymis. Depending upon morphological and functional differences, the epididymis is divided into 3 segments, caput (head), corpus (body), and cauda (tail) (Cosentino and Cockett, 1986).

A numerous factors, including steroid and peptide hormones, involve in the regulation of functions of the EDs and the epididymis. Of these factors, estrogen is known to play an important role in the EDs and the epididymis. A number of researches have shown the presence and expression of estrogen receptor (ER) α and ERβ during postnatal development (Cooke et al., 1991b; Lee et al., 2008; Sar and Welsch, 2000; Zhou et al., 2002). Lee et al. (2008) also demonstrated the presence of ERα and ERβ in the EDs throughout postnatal development period. Estrogen modulates the expression of genes involved in fluid reabsorption in the EDs (Lee et al., 2001; Lee et al., 2008; Oliveira et al., 2005). In the rabbit epididymis, estrogen, not androgen, regulates expression and activity of oxytocin receptor (Filippi et al., 2002). Estrogen also involves in modulation of expression of cytochrome P450 enzymes in the epididymis of the hamster (Hudson et al., 2001) and in alteration of protein profile in the epididymis of adult mice neonatally exposed to estrogen (Normand et al., 1990). Moreover, disruption of estrogen action in the male reproductive tract results in morphological and functional abnormalities in the EDs and the epididymis, as observed in ERα knockout (αERKO) and a pure anti-estrogen, ICI 182,780, treated mice (Cho et al., 2003; Hess et al., 1997; Hess et al., 2000; Lee et al., 2000; Lee et al., 2001). Consequently, these data indicate a significant role of estrogen and ERs for maintaining normal morphology and function of the male excurrent duct.

Estrogen is synthesized from an irreversible conversion of testosterone by the action of cytochrome P450 aromatase (*Cyp19*). Most of recent data show that major sources of estrogen in the male reproductive tract are germ cells, Leydig cells, and Sertoli cells in the testis and spermatozoa traversing the excurrent duct (Hess et al., 2001; Carreau et al., 1999). However, a growing body of evidence has shown that epithelial cells themselves of the EDs and the epididymis are capable of estrogen synthesis (Carpino et al., 2004; Pereyra-Martinez et al., 2001; Shayu and Rao, 2006; Wiszniewska, 2002). These researches indicate that the EDs and the epididymis act not only as functional tissues but also as endocrine organs for male reproduction.

Thus, in the present study, we attempted to determine expression of *Cyp19* gene in the EDs and the epididymis of the rat during postnatal development. In addition, we compared expression levels of *Cyp19* mRNA within each segment of the epididymis at different postnatal ages.

Ⅱ. MATERIALS AND METHODS

1. Isolation and collection of the tissue from animals

Male Sprague Dawley rats were purchased from Samtako (O San, S. Korea) and housed under controlled conditions and given *ad libitum* food and water until reaching proper ages. For the present study, we selected 4 experimental groups, 7 days (N = 8), 14 days (N = 6), 30 days (N = 6), and 90 days (N = 5) of ages. When an experimental animal became a proper age, rats

were anesthetized by CO_2 stunning. Male reproductive tracts were isolated, and the efferent ductules (EDs) and the epididymis were separated from the testis. The EDs were rapidly trimmed away from epididymal fat, and the epididymis was further dissected into 3 parts, caput (head), corpus (body), and cauda (tail). Tissues were washed with ice-cold PBS buffer before being frozen in liquid nitrogen. Because of the size of the EDs, the EDs isolated from an age group were pooled to get enough amount of total RNA for reverse transcription (RT) reaction and real-time polymerase chain reaction (PCR).

2. Total RNA isolation and Reverse transcription reaction

Total RNAs were isolated from the tissues by using easy-Blue total RNA extraction solution (iNtRON Biotech., Sungnam, S. Korea) and a Polytron homogenizer (Fisher Scientific, Pittsburgh, USA). The isolated RNA pellets were dissolved in RNA storage buffer (Ambion, Austin, USA) and stored at −80℃ until used for RT reaction. The purity and yield of the total RNAs were determined by an UV spectrophotometer (Eppendorf, New York, USA), and the qualities of the total RNAs were checked by gel electrophoresis prior to proceeding RT reaction. Oligonucleotide primers for PCR were prepared by utilizing published information. Information of primers of *Cyp19* and *GAPDH* tested for the present study are summarized in Table 1. The RT reactions were performed according to the instruction in ImProm-II™ reverse transcription system (Promega, Madison, USA). Briefly, 2 μg of isolated total RNAs was subjected for RT reaction in total volume of 20 μl using oligo-dT primer. RT reaction was carried out at 25℃ for 5 min, 42℃ for 1 hour, and 70℃ for 15 min.

3. Real-time PCR analysis

The real-time PCR procedures were performed according to the instructions in GoTaq DNA polymerase (Promega, Madison, USA). Briefly, the PCR mixture was consisted of 1 μl of cDNA, 0.75U of GoTaq DNA polymerase, 5 μl of 5X buffer, 0.2 mM of dNTPs (Promega, Madision, USA), 2.5 μl of 3000X SYBR Green (BMA, Rockland, USA), and 10 pmol of each primer. The PCR program employed an initial step of 95

Table 1. Primer sequences, expected product sizes, and PCR conditions for real-time PCR

Molecule	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')	Product size (bp)	PCR condition	GenBank accession number
Cyp19	GCTTCTCATCGCAGAGT ATCCGG (1555-1577)	CAAGGGTAAATTCATT GGGCTTGG (1821-1844)	290	94°C, 30 sec 65°C, 30 sec 72°C, 30 sec	M33986
GAPDH	CCCCTGGCCAAGGTCA TCCATGACAACTTT (540-569)	GGCCATGAGGTCCACC ACCCTGTTGCTGTA (1023-1052)	513	94°C, 30 sec 60°C, 30 sec 72°C, 30 sec	X02231

Cyp19 : cytochrome P450 aromatase
Numbers in parentheses of primer sequences indicate the positions of bases in GenBank sequences.

℃ for 5 min for denaturation, followed by denaturation at 94℃, annealing, and extension step at 72℃ of cycles. The PCR conditions for *Cyp19* and *GAPDH* were summarized in Table 1. The final extension at 72℃ for 10 min was carried out for the PCR. The PCR products were fractionized on 1.2% agarose gel to check the sizes. The image of each gel was photographed under UV using an image documentation system (Vilber Lourmat, Marne-la-Vallée, France). In this assay, we included *GAPDH*, which served as an internal PCR control.

4. Data presentation and statistical analysis

We repeated the RT reaction and real-time PCR for each age group at least three times to obtain a mean and a standard deviation. Expression level of *Cyp19* mRNA examined in the present study was normalized with its of *GAPDH*. The ratios of mRNA expression levels of *Cyp19* were expressed relative to 7 days of age for the EDs and 14 days of age for the epididymis as arbitrary unit. Mean differences among different age groups within the EDs and parts of the epididymis were compared using one-way ANOVA, followed by Tukey's test. In all cases, results were considered significant if $P < 0.05$.

Ⅲ. RESULTS

1. Expression of *Cyp19* mRNA in the EDs of male rats during postnatal period

Expression pattern of *Cyp19* transcript in the EDs is shown in Fig. 1. The transcript of *Cyp19* in the EDs was detected at all prepubertal ages, 7, 14, and 30 days of ages, but not at 90 days of age. A significant increase of *Cyp19* mRNA expression was found at 14 days of age, compared with the level of *Cyp19* transcript at 7 days of age (Fig. 1). However, the level of *Cyp19* mRNA was drastically decreased at 30 days of age, followed by no expression of *Cyp19* transcript at 90 days of age (Fig. 1).

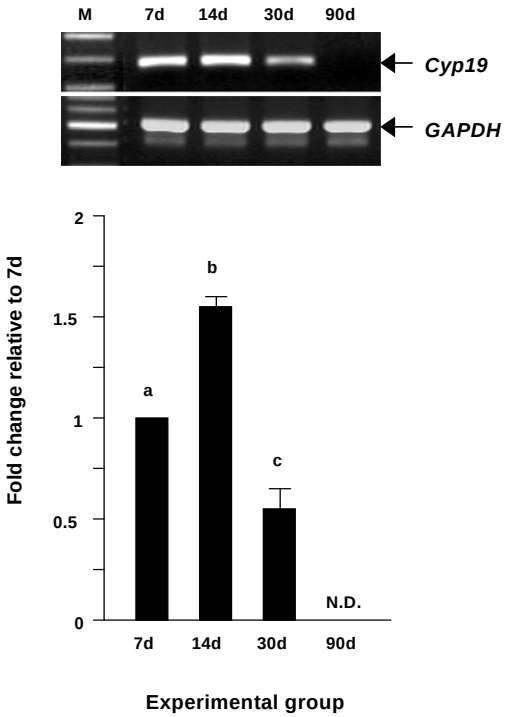

Fig. 1. Expression of *Cyp19* mRNA in the efferent ductules of rats during postnatal development. A representative photograph and a graph showing relative expression level of *Cyp19* transcript. M: 100bp marker. N.D.: not detectable. d: days of age after the birth. Different letters indicate significant differences among means ($P<0.05$).

2. Expression patterns of *Cyp19* mRNA in the epididymis of postnatally developing rats

Expression of *Cyp19* mRNA in the epididymis

was first detected at 14 days of age after the birth, regardless of segments of the epididymis (Fig. 2, 3, and 4). In the caput epididymis, compared with the level of *Cyp19* transcript at 14 days of age, a significant increase of *Cyp19* mRNA level was found at 30 days of age (Fig. 2). However, no significant change of *Cyp19* transcript level was detected at 90 days of age, compared with that at 14 days of age (Fig. 2).

Expression patterns of *Cyp19* mRNA in the corpus and caudal epididymis were different with that in the caput epididymis (Fig. 3 and 4). The lowest expression of *Cyp19* mRNA in the corpus epididymis was found at 30 days of age (Fig. 3). However, the level of *Cyp19* transcript was significantly increased at 90 days of age (Fig. 3). In the caudal epididymis, a surge of *Cyp19* mRNA expression was observed at 30 days of age (Fig. 4). The level of *Cyp19* transcript at 90 days of age was significantly lowered than that at 30 days of age, but still significantly higher than that at 14 days of age (Fig. 4)

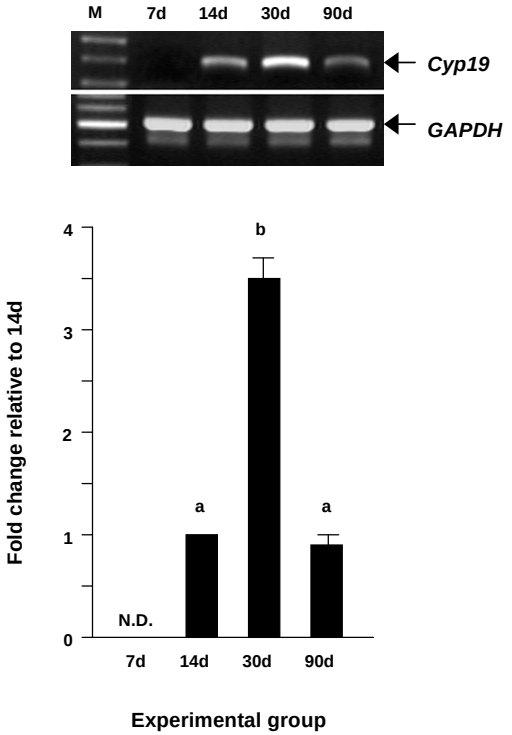

Fig. 2. Expression of *Cyp19* mRNA in the caput epididymis of rats during postnatal development. A representative photograph and a graph showing relative expression level of *Cyp19* transcript. M: 100bp marker. N.D.: not detectable. d: days of age after the birth. Different letters indicate significant differences among means (P<0.05).

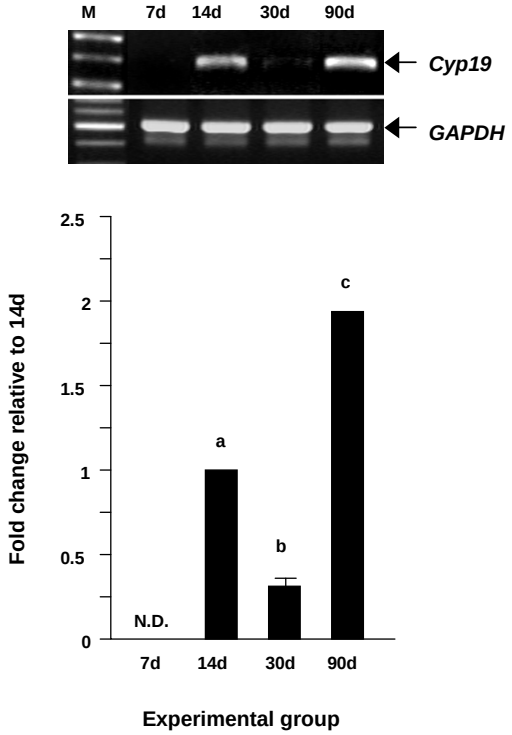

Fig. 3. Expression of *Cyp19* mRNA in the corpus epididymis of rats during postnatal development. A representative photograph and a graph showing relative expression level of *Cyp19* transcript. M: 100bp marker. N.D.: not detectable. d: days of age after the birth. Different letters indicate significant differences among means (P<0.05).

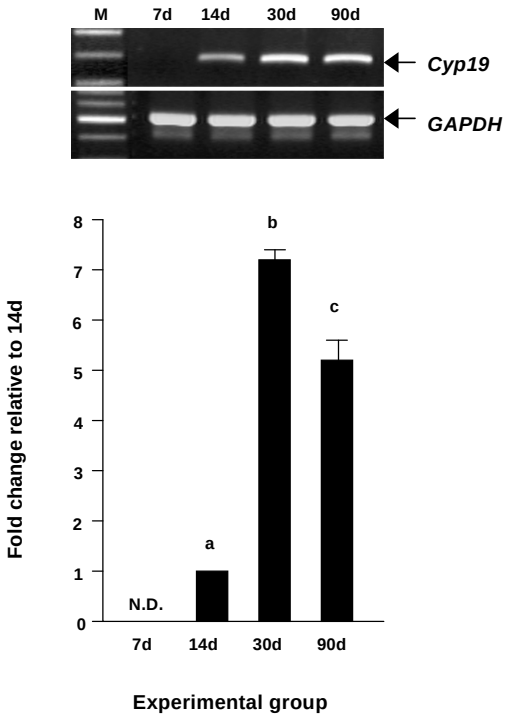

Fig. 4. Expression of *Cyp19* mRNA in the caudal epididymis of rats during postnatal development. A representative photograph and a graph showing relative expression level of *Cyp19* transcript. M: 100bp marker. N.D.: not detectable. d: days of age after the birth. Different letters indicate significant differences among means (P<0.05).

Ⅳ. DISCUSSION

The present study demonstrates the presence and differential expression of *Cyp19* transcript in the EDs and the epididymis of the male reproductive tract during postnatal development. Expression of *Cyp19* mRNA in the EDs was detected during prepubertal age and not at adult, while detectable level of *Cyp19* mRNA in the epididymis was appeared at 14 days of age and thereafter. In addition, the current study shows segment-dependent expression of *Cyp19* mRNA in the epididymis during postnatally developing period.

In the current study, we chose 4 different postnatal age groups based on feasible difference of secretion of the testicular fluid. It is extremely difficult to directly measure and analyze secretion of the testicular fluid in immature rats. Active secretion of the testicular fluid is indirectly indicated by the formation of the Sertoli cell junction which is occurred between 10 and 16 days of age (Gondos and Berndston, 1993; Nagano and Suzuki, 1976) and completed around 20 days of age (Martin and Dierichs, 1983). Thus, it is likely that secretion of the testicular fluid in the rat testis does not emerge at 7 days of age and actively occurs at 1 month of age. However, it is expected that full and active secretion of the testicular fluid would appear at 90 days of age.

The appearance of *Cyp19* transcript in the EDs of postnatally developing rats was first detected at 7 days of age. Because active secretion of the testicular fluid in rat would not begin until 10 days of age (Gondos and Berndston, 1993; Nagano and Suzuki, 1976), it is reasonable to consider that expression of *Cyp19* mRNA at very early postnatal development would be regulated by extra-testicular factor (s), rather than factor (s) present in the testicular fluid. An observation showing a significant increase of *Cyp19* transcript level at 14 days of age implies that the testicular factor (s) released upon the formation of the Sertoli cell junction would involve in induction of *Cyp19* mRNA expression in the EDs. However, we can not rule out a possibility of the action of extra-testicular factor (s) which influences on gene expression of *Cyp19* at this age. Interestingly, expression of *Cyp19* gene was

significantly decreased at 30 days of age, and furthermore, *Cyp19* transcript was not detected at 90 days of age. These results indicate that the testicular factor(s) released from the testis would involve in down-regulation and/or inhibition of *Cyp19* mRNA expression at near puberty and adulthood.

There was no expression of *Cyp19* mRNA at 7 days of age, regardless of segments of the epididymis. The first appearance of *Cyp19* mRNA in the entire epididymis was at 14 days of age, in close agreement with the finding of Shayu and Rao (2006) which shows the presence of *Cyp19* transcript in the caput and corpus epididymis at 20 days of age. However, it is not clear at this point if the testicular factor (s) influences on the expression of *Cyp19* at this age. Because the formation of Sertoli cell junction in rat occurs between 10 and 16 days of age (Gondos and Berndston, 1993; Nagano and Suzuki, 1976) and is not completed until 20 days of age (Martin and Dierichs, 1983), it is hard to consider that the testicular fluid gives a great influence on *Cyp19* mRNA expression in the epididymis at 14 days of age. Thus, expression of *Cyp19* mRNA in the epididymis at 14 days of age is likely induced by extra-testicular factor(s) and/or combination with the testicular factor (s), if any. Further investigation is required to determine factor (s) involving in regulation of *Cyp19* mRNA expression in the epididymis at the early postnatal development.

At near puberty and adult, expression of *Cyp19* mRNA in the epididymis was differentially regulated in segment-specific manner. In the caput, the level of *Cyp19* mRNA was significantly increased at 30 days of age, but was not changed at 90 days of age, compared with that at 14 days of age. In contrast, the corpus epididymis had the lowest expression of *Cyp19* mRNA at 30 days of age but the highest expression at 90 days of age. In the caudal epididymis, the levels of *Cyp19* mRNA at these ages were significantly higher than that at 14 days of age. It is not evident why the level of *Cyp19* mRNA in the epididymis shows distinct segmental patterns after 14 days of age. We speculate that such differential expression of *Cyp19* mRNA among segments of the epididymis would be a result of combined effect of testicular and/or extra-testicular factor (s). Indeed, Shayu and Rao (2006) showed that androgens and luteinizing hormone (LH) involve in modulation of *Cyp19* mRNA expression and activity in the epididymis. In addition, they demonstrated the differential expression of LH receptor between the caput and caudal epididymis (Shayu and Rao, 2006). Moreover, testosterone concentration rises at 39~41 day which is about 10 days after a significant rise in the concentration of the serum LH (de Jong and Sharpe, 1977). Based on these previous findings, therefore, we presume that changes of testosterone concentration and LH receptor expression during postnatal development would result in segmental-dependent expression of *Cyp19* mRNA in the epididymis, as observed in the present study. To determine a precise regulatory mechanism of *Cyp19* mRNA expression in the epididymis, additional researches are required to evaluate expression patterns of androgen receptor and LH receptor among segments of the rat epididymis during postnatally developing period.

There are numbers of researches demonstrating in temporal and sequential appearance of gene expression along the excurrent duct of male reproductive tract during the developmental period. The first appearance of androgen receptor (AR) is found in the EDs within the excurrent

duct, and the caput epididymis has higher AR level than the caudal epididymis (Cooke et al., 1991a). In addition, there is a gradient of ERα mRNA level along the excurrent duct, which is the highest level of ERα in the EDs and the lowest expression of ERα in the caudal epididymis (Cooke et al., 199b). In the present study, the expression of *Cyp19* mRNA in the excurrent duct is first detected in the EDs with the highest level at 14 days of age. Even though a direct comparison of *Cyp19* mRNA levels among the epididymal segments is not possible in the present study, there seems to be age-dependent sequential *Cyp19* mRNA expression among the epididymis. That is, the highest level of *Cyp19* mRNA is detected in the caput epididymis at 30 days of age and in the corpus epididymis at 90 days of age. In the caudal epididymis, significant increases of *Cyp19* mRNA levels are found at 30 and 90 days of ages. We have no clear explanation for such age- and segment-dependent differential expression of *Cyp19* mRNA expression in the male excurrent system. However, it is presumed that expression of *Cyp19* mRNA in the excurrent duct is closely related with functional regulation of the EDs and epididymis, so thus sustaining male fertility after the puberty.

In conclusion, to our knowledge, this is the first study to reveal a differential expression of *Cyp19* mRNA in the rat EDs and the epididymis in age-dependent and segment-specific manners. Also, the data from the present study provides a prospect that the EDs and epididymis would function as endocrine organs in the male reproductive tract. Detailed studies are suggested to resolve a functional role of CYP19 in the EDs and epididymis.

Ⅴ. ABSTRACT

The present study was performed to determine expression of cytochrome P450 aromatase (*Cyp19*) in the efferent ductules (EDs) and the epididymis of male rat reproductive tract at different postnatal ages. Total RNAs isolated were reverse-transcribed, and cDNAs were utilized for real-time PCR analysis. In the EDs, the *Cyp19* transcript was expressed at all prepubertal ages with the highest level at 14 days of age, but not at 90 days of age. Expression of *Cyp19* mRNA in the epididymis was found at all age groups, except 7 days of age. Distinct expression patterns of *Cyp19* transcript were shown in each segment of the epididymis. These results indicate that expression of *Cyp19* gene in the excurrent duct of male reproductive tract is differentially regulated in age-dependent and segment-specific manners.

(**Key words** : Efferent ductules, Epididymis, Cytochrome P450 Aromatase, mRNA expression)

Ⅵ. ACKNOWLEDGEMENT

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