

The Expression Patterns of Connexin Isoforms in the Rat Caput Epididymis During Postnatal Development

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ABSTRACT

Intercellular interactions are important for the proper development and regulation of tissue function. This is especially necessary in the epididymis, a part of male reproductive tract where sperm become mature and acquire their fertilizing capacity. The caput region of the epididymis consists of several types of cells, including principal, basal, and apical cells. Direct intercellular communication is thus required to precisely regulate the functions of the caput epididymis. In this regard, connexin (Cx) is a molecule that forms channels, which allow the direct exchange of small molecules between cells, enabling intercellular communication. In this study, the expression of Cx isoforms in the caput epididymis at different postnatal ages was determined by using quantitative real-time polymerase chain reaction analysis. Nine of 13 Cx isoforms were detected. The transcript levels of Cx30.3, 31, 31.1, 32, and 40 were highest at 45 days of age, while the expression of Cx43 and 45 gradually decreased with age. A substantial fluctuation of Cx26 expression was detected, with significant decreases before and during puberty, followed by a transient increase at adult-hood and rapid decreases at an old age. A significant increase in Cx37 transcript was observed at 25 days of age, followed by gradual decreases at adult and old ages. These results indicate the significant differential expression of various Cx isoforms in the caput epididymis during postnatal development. It further suggests that the functional regulation and developmental maturation of the caput epididymis are highly related to the postnatal age-related differential expression of Cx isoforms.

(Key words : Connexin, Caput epididymis, Gene expression, Development, Male)

INTRODUCTION

The epididymis is a part of the male reproductive excurrent ductal system, between the efferent ducts and the vas deferens. The epididymis consists of a single coiled tubular duct and has a polarized epithelial layer surrounded by a smooth muscle layer (Sullivan, 2004). During embryonic development, under the influence of androgens secreted from the male gonad, the Wolffian ducts develop into a number of male reproductive organs, such as the epididymis, vas deferens, and seminal vesicles (Hannema and Hughes, 2007). In general, the differentiation of epididymal epithelial cells appears in two developmental phases during postnatal development a first phase in the early neonatal period, in which halo cells appear from undifferentiated cells, and a second phase, in which there is a sequential differentiation of other cell types until adulthood (Arrotéia et al., 2012). It is believed that a number of lumicrine factors produced by the

testis, such as androgens, growth factors, and other secretory molecules, play important roles in the differentiation of the epithelium (Hinton et al., 2011).

Based on anatomical and functional differences, the epididymis is divided into four segments, the initial segment, caput epididymis, corpus epididymis, and cauda epididymis (Lasserre et al., 2001). Each epididymal segment is confined by connective tissue septa to functionally separate the epididymal regions (Cornwall, 2009). The epithelium of the epididymis is composed of several cell types, including principal, basal, apical, narrow, clear, and halo cells (Arrotéia et al., 2012). Each region of the epididymis has a different cellular organization with distinct cell populations, although the principal cell is the most abundant cell type throughout the entire epididymal duct (Arrotéia et al., 2012). For example, the number of principal cells decreases from 80% of the total epithelial cell population in the initial segment to 65% in the cauda epididymis (Robaire and Hermo, 1988).

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However, the number of narrow cells in the total epithelial cell population increases along the epididymis from 3% in the initial segment to 6% in the corpus epididymis (Arrotéa et al., 2012). In addition, each cell type in the epididymal epithelium has different roles that influence overall epididymal functions. The chief function of the principal cell is the secretion of a bulk of proteins to regulate luminal protein concentrations (Robaire et al., 2000), while the narrow cell is involved in the intracellular transport of specific molecules between the lumen and epithelial cells (Robaire et al., 2006). The clear cell is likely responsible for the removal of luminal proteins, and the apical cell is related to the regulation of luminal pH (Hermo et al., 2005). It is reasonable to consider that different cellular compositions along the epididymis would lead to the creation of a specific luminal microenvironment in each epididymal region, resulting in the regionalization of epididymal functions.

The main functions of the epididymis include transport, storage, maturation, and concentration of spermatozoa for male fertility. To accomplish these many functions, the epididymis is required to establish a particular microenvironment through the secretion and absorption of specific proteins and ions at different regions. Because there are several cell types in the epididymal epithelium, a precise regulation of intercellular communication is highly essential to control the functions of the epididymis. This communication requires gap junctional complexes formed by connexin (Cx) molecules (Goodenough and Paul, 2009). There are at least 20 Cx isoforms in rodents, and the gap junctional complexes allow intercellular interactions by controlling the transfer of small molecules among different cell types (Goodenough and Paul, 2009). In the epididymis, the expression of several Cx molecules, including Cx26, 30.3, 31.1, 32, and 43, during the early postnatal period has been determined (Dufresne et al., 2003). In addition, the expression patterns of these Cx molecules within the head part (initial segment, caput epididymis, and corpus epididymis) and tail part of the epididymis have been examined (Dufresne et al., 2003). However, the presence of other Cx isoform transcripts in the epididymis has not been revealed. Moreover, the definite regional comparison of Cx isoform expression has not been attempted.

In the present study, the existence of Cx isoforms transcripts in the caput epididymis was examined by quantitative real-time PCR analysis. In addition, the expression patterns of Cx isoforms in the caput epididymis,

from the neonatal to old age, during the postnatal period were determined and compared with previous research findings.

MATERIALS AND METHODS

1. Experimental animals and tissue isolation

Male Sprague Dawley rats at different postnatal ages were used for the present study. Neonatal rats at 1 week (n=10) and 15 days (n=10) of ages were obtained from pregnant female rats purchased from Samtako (O San, S. Korea). Live animals at 25 days (n=8) and 45 days (n=7) of age were also obtained from Samtako. Free access to food and drinking water were permitted for experimental animals. Adult animals at 5 months (n=5) and 1 year (n=3) of age and elderly animals at 2 years (n=3) of age were kindly provided by the Aging Tissue Bank (Department of Pharmacology, Pusan National University, S. Korea).

CO₂ stunning was employed to anesthetize experimental animals at proper ages. The lower abdominal area was then exposed, and the testis and epididymis were dissected out. The epididymis was separated from the testis in cold phosphate buffered saline (PBS). The epididymis was further cut into the initial segment, caput epididymis, corpus epididymis, and cauda epididymis based on information from previous research (Guo et al., 2007; Robaire et al., 2006). The caput epididymis at 1 week, 15 days, or 25 days of age was pooled because each caput epididymal segment was too small to acquire sufficient amounts of total RNA for real-time PCR analysis. The caput epididymis was rapidly frozen in liquid nitrogen and kept at -80°C until the isolation of total RNA.

2. Extraction of total RNA from the caput epididymis and preparation of complementary DNA (cDNA)

The frozen caput epididymis was homogenized with a polytron homogenizer (Fisher Scientific, Pittsburgh, PA) in an Eppendorf tube with easy-Blue total RNA extraction solution (iNtRON Biotech, Sungnam, S. Korea). Using the phenol-chloroform extraction method, a RNA pellet was obtained. The RNA was dissolved in RNA storage buffer (Ambion, Austin, TX) and then stored at -80°C until the generation of first strand cDNA. The concentrations of total RNA were measured by an UV spectrophotometer (Eppendorf, New

York, NY). The qualities of total RNA were checked by gel electrophoresis.

Onemicrogram of total RNA was used for a reverse transcription (RT) reaction to generate the first stranded cDNA in the ImProm-IITM reverse transcription system (Promega, Madison, WI). The RT reaction was carried out with oligo-dT primer in a total volume of 20 μ L. The RT reaction was performed at 25°C for 5 min, 42°C for 1 hr, and 70°C for 15 min. The generated cDNAs were stored in -20°C until real-time PCR analysis.

3. Quantitative real-time polymerase chain reaction (PCR) analysis

Table 1 shows the oligonucleotide primers used for real-time PCR analysis. In preliminary examinations, the expression of 13 Cx isoforms (Cx26, 30, 30.3, 31, 31.1, 32, 33, 36, 37, 40, 43, 45, and 50) in the caput epididymis was tested. However, only nine transcripts (Cx26, 30.3, 31, 31.1, 32, 37, 40, 43, and 45) of 13 Cx isoforms were detected, and the expression patterns of these Cx isoforms were determined in the present study.

To conduct real-time PCR analysis, a mixture of 1 μ L of cDNA, 10 pmol of primer set, and 10 μ L of master mix (Finnzymes, Espoo, Finland) was prepared, and nuclease

free-water was added to a final volume of 20 μ L. A pre-denaturation step of the PCR mixture was carried out at 95°C for 5 min to form single-strands from the cDNA template. The PCR procedure was then followed by a number of cycles of denaturation at 95°C for 30 sec, annealing at T_m for 30 sec, and extension at 72°C for 30 sec. An extra extension step at 72°C for 10 min was added at the end of the PCR. Cyclophilin A (*Ppia*) was used as an internal quantitative control for PCR. All PCR products were examined through agarose gel electrophoresis to check the size of the PCR products.

4. Data collection and statistical analysis

Independent and quadruplicate RT-PCR reactions were conducted to obtain a mean and standard error for each age group. The expression levels of Cx molecules were normalized to the expression of *Ppia*. Real-time PCR results were expressed in relative expression ratios between Cx and *Ppia*. Statistical significance of expression levels for each Cx isoform among different postnatal age groups was determined by one-way ANOVA, followed by Duncan's test, a post-hoc analysis. When the P value was lower than 0.05, results were regarded as statistically significant.

Table 1. List of oligonucleotide primers utilized for real-time PCR analysis

Gene (GenBank ID)	Primer sequence (5' → 3')	T _m (°C)	PCR product size (bps)
Cx26 (NM_001004099)	(F) TCCTCTCATCTCCGCATC (R) CCGTTTCTTTTCGTGTCTCC	55	233
Cx30.3 (NM_053984)	(F) CCAATGTCTGCTATGACGA (R) CACAGCAGCCTTGAAGATGA	57	243
Cx31 (NM_019240)	(F) TTGAGCGGTGTGAACCAGTA (R) TGTTGGAGATGGGGAAGAAG	57	193
Cx31.1 (NM_019241)	(F) CATCGTCTGCATCCTGCTTA (R) ATGAGGTCGCTTGAGAGGAA	55	165
Cx32 (NM_017251)	(F) AGAATCATGGTGCTGGTGGT (R) CCTCAAGCCGTAGCATTTC	57	235
Cx37 (NM_021654)	(F) AGTGTCTGTACCTTGGATGCC (R) CAGCACACTTAGCCAAGAGC	51	223
Cx40 (NM_019280)	(F) ATACCATTTCAGCCTGGTTGC (R) CGGCCTCTTTAGCTTTCTCA	57	189
Cx43 (NM_012567)	(F) AGCAAGCTAGCGAGCAAAC (R) GAGTTCATGTCCAGCAGCAA	55	151
Cx45 (NM_001085381)	(F) GATCATCTGGTTGCTACTC (R) GATCCTCTTCATGGTCCTCT	51	173
<i>Ppia</i>	(F) GGCAATGCTGGACCAAACAC (R) TTAGAGTTGTCCACAGTCGGAGATG	59	196

Cx : connexin; *Ppia* : peptidylprolyl isomerase A (cyclophilin A).

RESULTS

1. Expression patterns of Cx26, Cx30.3, and Cx31 in the rat caput epididymis during the postnatal period

The expression pattern of Cx26 is shown in Fig. 1a. Expression levels of Cx26 were higher at 1 week of age

(neonatal period) and 15 days of age than at 25 days of age and 45 days of age. However, a transient increase of Cx26 transcript level was observed in the caput epididymis at 5 months of age, followed by a significant drop of level at 1 year of age (Fig. 1a). There was no expression change between 1 year of age and 2 years of age (Fig. 1a).

Minimal expression of the Cx30.3 gene in the caput

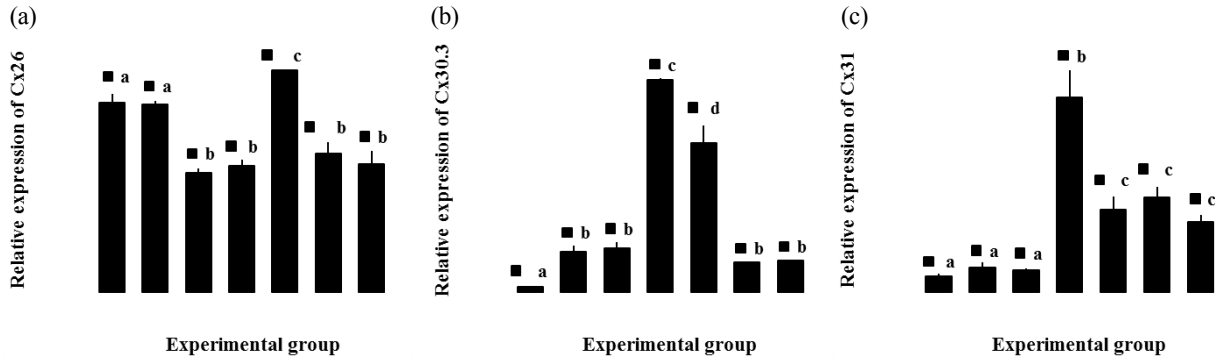


Fig. 1. Expression patterns of Cx26, Cx30.3, and Cx31 in the rat caput epididymis during the postnatal period. The relative expression levels of Cx26 (A), Cx30.3 (B), and Cx31 (C) are shown. Statistically significant differences ($p < 0.05$) are indicated in different letters. D: day, M: month, W: week, and Y: year.

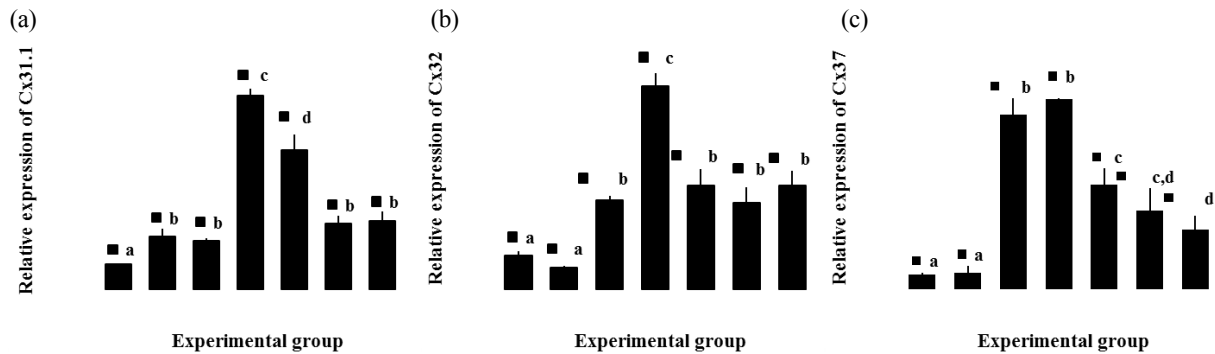


Fig. 2. Expression patterns of Cx31.1, Cx32, and Cx37 in the rat caput epididymis during the postnatal period. The relative expression levels of Cx31.1 (A), Cx32 (B), and Cx37 (C) are shown. Statistically significant differences ($p < 0.05$) are indicated in different letters. D: day, M: month, W: week, and Y: year.

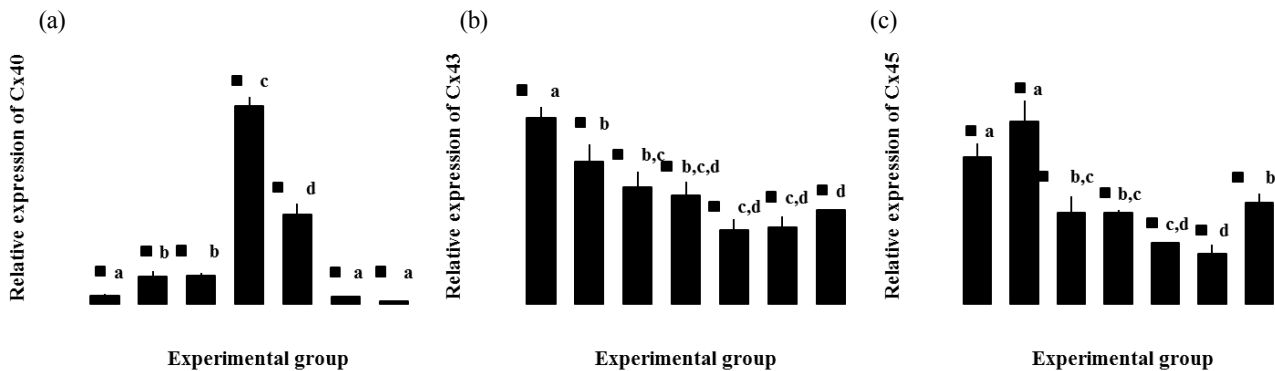


Fig. 3. Expression patterns of Cx40, Cx43, and Cx45 in the rat caput epididymis during the postnatal period. The relative expression levels of Cx40 (A), Cx43 (B), and Cx45 (C) are shown. Statistically significant differences ($p < 0.05$) are indicated in different letters. D: day, M: month, W: week, and Y: year.

epididymis was found at 1 week of age (Fig. 1b). However, the gene expression of Cx30.3 significantly increased at 15 days of age, compared with 1 week of age (Fig. 1b). There was no difference in Cx30.3 transcript levels between 15 days of age and 25 days of age, while the Cx30.3 transcript level drastically increased at 45 days of age (Fig. 1b). The expression of Cx30.3 in the caput epididymis significantly decreased at 5 months of age, compared to 45 days of age (Fig. 1b). The subsequent decrease in Cx30.3 gene expression was detected at 1 year of age, and the expression levels of Cx30.3 between 1 year of age and 2 years of age were not statistically different (Fig. 1b).

Cx31 gene expression did not change during the early postnatal period, but a significant increase in Cx31 expression was observed at 45 days of age (Fig. 1c). A dramatic decrease in Cx31 expression was then followed at 5 months of age, with no significant change in Cx31 mRNA levels at adult and old ages (Fig. 1c).

2. Expression patterns of Cx31.1, Cx32, and Cx37 in the rat caput epididymis during the postnatal period

The expression pattern of Cx31.1 was had similarities to the expression pattern of Cx30.3. The first significant increase of Cx31.1 gene expression was detected at 15 days of age (Fig. 2a). An additional rise in Cx31.1 transcript level was observed at 45 days of age, followed by a significant decrease at 5 months of age (Fig. 2a). At older ages (1 year and 2 years), the abundance of Cx31.1 mRNA was significantly lower than at 5 months of age (Fig. 2a).

There was no significant difference in Cx32 transcript levels between 1 week of age and 15 days of age (Fig. 2b). However, the abundance of Cx32 mRNA greatly increased at 25 days of age, and the highest level of Cx32 transcripts was detected at 45 days of age (Fig. 2b). After a transient decrease of Cx32 mRNA level at 5 months of age, there was no significant change of Cx32 abundance in the caput epididymis during the elderly period (Fig. 2b).

Minimal expression of the Cx37 in the caput epididymis was found at early postnatal ages, 1 week of age and 15 days of age (Fig. 2c) however, a surge in Cx37 expression was detected at 25 days of age (Fig. 2c). The abundance of Cx37 mRNA did not significantly change at 45 days of age, compared with 25 days of age (Fig. 2c). The level of Cx37 transcripts significantly dropped at 5 months of age, followed

by gradual decreases of Cx37 mRNA levels at 1 year of age and 2 years of age (Fig. 2c).

3. Expression patterns of Cx40, Cx43, and Cx45 in the rat caput epididymis during the postnatal period

Expression of the Cx40 gene significantly increased at 15 days of age, compared with 1 week of age (Fig. 3a). A tremendous increase of Cx40 gene expression was detected at 45 days of age, followed by a transient decrease at 5 months of age (Fig. 3a). Cx40 gene expression at 1 year of age and 2 years of age was minimal (Fig. 3a).

Expression patterns of Cx43 in the caput epididymis during postnatal development are shown in Fig. 3b. Cx43 gene expression was highest at 1 week of age, followed by a gradual decrease with age (Fig. 3b). After puberty, there was no significant change of Cx43 mRNA level in the caput epididymis (Fig. 3b). The abundance of Cx45 transcripts was significantly higher at 1 week of age and 15 days of age, compared with other ages (Fig. 3c). There was a steady decrease of Cx45 gene expression until 1 year of age, followed by an increase at 2 years of age (Fig. 3c).

DISCUSSION

Direct intercellular communication through Cx is important for the regulation and maintenance of specific tissue functions. The current research demonstrates the presence of a number of Cx isoforms in the caput epididymis of the rat. In addition, the differential expression of Cx isoforms during the postnatal period has been revealed by real-time PCR analysis.

The existence of various Cx isoforms in the epididymis has been previously observed (Cyr et al., 1996; Dufresne et al., 2003). However, no research has been conducted to determine the expression patterns of Cx isoforms in defined epididymal regions at multiple developmental stages. The present study examined the expression of Cx isoforms in the rat caput epididymis during the postnatal period. Dufresne et al. (2003) have shown the expression of Cx26, 30.3, 31.1, and 32 in the caput epididymis and the expression of Cx43 in the adult caput epididymis (Cry et al., 1996). In our research, in addition to finding these Cx isoforms, the expression of Cx31, 37, 40, and 45 in the caput epididymis was detected. These findings indicate possibility of the existence of other Cx isoforms, except 13 Cx isoforms tested

in the present study. Indeed, the expression of particular Cx isoform in various tissues is frequently observed (Bosco et al., 2011). Thus, the existence of other Cx isoforms should be investigated in future studies.

The localization of Cx isoforms in the epididymis has been studied (Cyr et al., 1996; Dufresne et al., 2003). Cx43 is between the principal and basal cells in the caput epididymis and does not exist between adjacent principal cells (Cyr et al., 1996). In the cauda epididymis, Cx43 also exists between clear and basal cells and between myoid cells surrounding the epididymal duct (Cyr et al., 1996). The immuno-localization of Cx32 in the epididymis is detected between adjacent principal cells and between principal and basal cells (Dufresne et al., 2003). In addition, the localization of Cx26 at the apical region of the epididymal epithelial cells is found in the early postnatal animal (Dufresne et al., 2003). Moreover, the co-localization of Cx26 and 32 between principal cells is detected in the epididymis (Dufresne et al., 2003). The expression of multiple Cx isoforms, as shown in the present study, implies the possibilities of the localization of more Cx isoforms in a specific caput epithelial cell than previous observations.

Differentiation of the epididymal epithelium into different cell types does not occur until 14 days after birth (Sun and Flickinger, 1979). After full differentiation of the epididymis until 45 days of age, the expansion of differentiated epididymal cells takes place between 45 days and 3 months of ages (Sun and Flickinger, 1979). Overall findings suggest the differential expression of Cx isoforms during the postnatal period. Indeed, our present study shows the expression change of several Cx isoforms in the caput epididymis at different postnatal ages. Dufresne et al. (2003) have also demonstrated similar findings in the rat epididymis. Interestingly, significant increases in the transcripts of Cx30.3, 31, 31.1, 32, and 40 in the caput epididymis were detected in our research at 45 days of age. Because the end of differentiation and the beginning of epididymal cell expansion occur at 45 days of age, we speculate that this expression increase is related to the maturation of epididymal cells during the postnatal period. A detailed examination on the development of epididymal cells near the early pubertal period requires future investigation.

The hormonal regulation of Cx43 gene expression in the epididymis has been examined in previous research. Chemically-induced hypothyroidism at a neonatal age results

in the decrease of Cx43 mRNA in the proximal epididymis (St-Pierre et al., 2003). Treatment with flutamide, an anti-androgen, leads to a decreased Cx43 expression in the cauda epididymis of adult boar, indicating possible androgen-dependent regulation of Cx43 expression in the epididymis (Lydka et al., 2011). In the stallion, the cryptorchid results in a significant decrease of Cx43 expression (Hejmej et al., 2007). Recently, Dubé et al. (2012) have reported the regulation of Cx43 expression by epidermal growth factor in the human epididymis. These observations imply that the expression of Cx genes in the epididymis is precisely regulated by various factors, including testicular factor(s). The serum testosterone concentration of the male rat is significantly increased between 40 and 50 days of age (Brown-Grant et al., 1975). Thus, it is possible that increases in Cx30.3, 31, 31.1, 32, and 40 gene expression in the caput epididymis at 45 days of age is influenced by testicular testosterone and/or growth factors.

It is notable that a significant induction of Cx37 mRNA level is observed at 25 days of age, indicating that Cx37 expression is regulated by molecular mechanism(s) distinct from other Cx isoforms. In addition, transcript levels of Cx43 and 45, at 45 days of age, are greatly lower than transcript levels at the early neonatal period. These observations indicate that Cx43 and 45 may play an important role in the differentiation and maturation of the caput epididymis during the early postnatal phase. At this point, it is uncertain what causes high levels of Cx43 and 45 gene expressions in the caput epididymis of the neonatal rat molecular factor(s) involved in the early postnatal development of the epididymis potentially influence Cx43 and 45 gene expression. Together, these results suggest that the transcriptional regulation of Cx isoforms in the caput epididymis is highly complicated and requires the coordinated actions of various factors at each period of postnatal development.

In conclusion, the functional maturation and development of the rat caput epididymis is associated with the expression of a number of Cx isoforms at distinct postnatal stages. Aging could influence the gene expression of Cx isoforms in the caput epididymis and may also lead to functional alterations in the caput epididymis of the aged male. The castration and/or efferent ductal ligation would provide valuable information of the effect of androgen on the expression of Cx isoform in the caput epididymis.

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