Expressional Profiling of Connexin Isoforms in the Initial Segment of the Male Reproductive Tract during Postnatal Development

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ABSTRACT

Functional regulation of a specific tissue or organ is controlled by a number of ways, including local cell-cell interaction. Of several forms of cell-cell junctional complexes, gap junctions are caught a great attention due to a formation of direct linkage between neighboring cells. Gap junctions are consisted of connexin (Cx) isoforms. In the present study, we evaluated expressional profiling of *Cx* isoforms in the rat initial segment (IS) of the male reproductive tract at different postnatal ages. The presence and expression of 13 *Cx* isoform mRNAs were determined by semi-quantitative real-time PCR analyses. A total of 8 *Cx* isoform mRNAs were detected in the IS of the male rats during postnatal development. The highest level of *Cx*30.3 mRNA was found at 5 months of age, while abundance of *Cx*31 mRNA was the highest at 1 year of age. Expression of *Cx*31.1 gene was relatively consistent during the postnatal development. Fluctuation of *Cx*32 and 37 gene expression was observed during the postnatal period. Significant elevation of *Cx*40 mRNA abundance was detected at 25 days of age and older ages. Expression patterns of *Cx*43 and 45 genes were similar with the highest level at 2 weeks of age, followed by gradual decreases at older ages. These results indicate differential regulation on expression of *Cx* isoforms in the rat IS during postnatal development. A complicated regulation of gene expression of *Cx* isoforms in the IS at different postnatal ages is suggested.

(Key words : Initial segment, Connexin, Postnatal development, Male reproduction, Gene expression)

INTRODUCTION

The male fertility is precisely regulated by combinational actions of intrinsic and extrinsic factors. Acquirement of fertilizing capacity of spermatozoa produced from the testis occurs while spermatozoa travel throughout the excurrent ducts of the male reproductive tract, including the efferent ductules and epididymis (Cornwall, 2009). The epididymis has a tubular structure, consisting of a single layer of epithelium surrounded by smooth muscle cells and connective tissues (Robaire and Hermo, 1988). Based on morphological and histological characteristics, the epididymis is divided into four regions, initial segment (IS), caput epididymis (head of epididymis), corpus epididymis (body of epididymis), and caudal epididymis (tail of epididymis) (Cosentino and Cockett, 1986). Differentiation of the epididymis derived from the Wolffian duct is completed by puberty and is dependent on androgens, as well as luminal factors secreted from the testis (Rodriguez *et al.*, 2002). Compartmentalization of the epididymis into different regions is thought to be regulated by regional-specific expression of a number of genes, including *Hox* genes, during postpartum development (Rao and Wilkinson, 2002). It is believed that such differential gene expression is closely related with creation of unique microenvironment of the fluid within each segment of the epididymis (Li *et al.*, 2008; Thimon *et al.*, 2008).

Formation of distinct physiology of each epididymal segment is likely resulted in a complex coordination of

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function by epididymal epithelium, indicating an importance of intracellular communication to epididymal function (Cyr *et al.*, 2002). Direct local cell-cell interactions are crucial in the regulation of physiological function in mammalian tissues, including the testis and the excurrent ducts of the male reproductive tract (Pointis *et al.*, 2005). These interactions include cadherin-based adhesion, occludin-based attachment, and connexin (Cx)-based gap junctions. Of these interactions, gap junction has caught a great attention because of direct intercellular exchange of molecules between the cytoplasm of two adjacent cells (Goodenough *et al.*, 1996). There are at least 20 Cxs identified up to date (Willecke *et al.*, 2002). A number of researches have shown that the expression of Cxs is in a tissue-specific manner and several cell types possess more than one type of Cxs (Goodenough *et al.*, 1996; Willecke *et al.*, 2002). Expression of certain Cxs is detected in various cell types, while other Cxs have shown a limited presence in specific cells and/or tissues (Pointis *et al.*, 2005). Even, others have demonstrated differential expression of Cxs in the male reproductive tract during postpartum development, such as Cx43 in the testis (Perez-Amendariz *et al.*, 2001) and multiple Cxs in the epididymis (Dufresne *et al.*, 2003) and the efferent ductules (Lee *et al.*, 2007). Moreover, Dufresne *et al.* (2003) have shown the segmental-specific expression of a number of Cxs in the epididymis during postnatal development. Together, results from these researches indicate that expression of Cxs in each part of the male reproductive tract is differentially regulated at different postnatal ages. However, detailed information on gene expressional regulation in specific parts of the male excurrent duct has not been elucidated yet.

The IS is widely considered as a part of the caput epididymis. However, the IS has a number of structural and cellular features distinguishable from the caput epididymis. The IS has a much thicker columnar epithelium than its of the caput epididymis (Robaire and Hermo, 1988). The epithelium of the IS is consisted of 5 different cell types, principal, narrow, apical, basal, and halo cells (Adamali and Hermo, 1996; Robaire and Hermo, 1988). While principal, basal, and halo cells are frequently found throughout the epididymis, narrow and apical cells are exclusively present in the IS (Adamali and Hermo, 1996; Robaire and Hermo, 1988). In addition to the existence of specialized cells types, cellular composition of these cell types in the IS differs from other segments of the epididymis (Robaire and Hermo, 1988). Moreover, histochemical analyses have demonstrated that the IS epithelium has distinct localization of enzymatic molecules, indicating that the IS carries out different functions distinctive from the rest of the epididymal regions (Adamali and Hermo, 1996). In fact, a number of researches have revealed different luminal compositions in the IS, compared with other epididymal segments (reviews in Robaire and Hermo, 1988). Collectively, these findings suggest that the function of the IS is regulated in different manners from the other epididymal regions. However, it has not been determined how functions of the IS are regulated at cellular level.

In the present study, using semi-quantitative real-time PCR analysis, we first attempted to determine expressional profiling of Cxs in the IS. Next, we evaluated expressional patterns of Cxs present in the IS at different ages during postnatal development. We found differential expression of a number of Cxs in the IS at different postnatal ages. These findings suggest that different cellular communication during postnatal development achieved by different forms of Cxs would relate with a proper maturation of the IS, so thus functions of the IS.

MATERIALS AND METHODS

Experimental Animals and Tissue Collection

Male Spragure Dawley rats were used for the present study. Considering a number of developmental significances during postnatal period, such as the times of junctional formation of the Sertoli cells in the testis, secretion of the testicular fluid, and fertilizing capacity, we chose 7 different age groups, 3 prepubertal ages (1 week, 2 weeks, and 25 days of ages), 1 pubertal age (45 days of age), 1 adult age (5 months of age), and 2 old ages (1 year and 2 years of ages). The prepubertal rats were obtained from delivery of pregnant female rats. All experimental animals were purchased from Samtako (O San, S. Korea) and housed under controlled conditions with *ad libitum* food and water supplies. Once reaching at proper ages, rats were anesthetized by CO_2 stunning, and the male reproductive tract was isolated. In cold PBS buffer, the epididymis was separated from the rest of the male reproductive tract. The initial segment (IS) was further dissected out from the caput epididymis and briefly washed in a new cold PBS buffer before quickly frozen in liquid nitrogen. The IS was stored in −80℃ until used for total RNA isolation later.

Total RNA Isolation and cDNA Generation

Total RNA from the collected IS was isolated using easy-Blue total RNA extraction solution (iNtRON Biotech, Sungnam, S. Korea). Tissue homogenization was performed with a polytron homogenizer (Fisher Scientific, Pittsburgh, USA). Phenol and Chloroform were subsequently added to obtain total RNA pellets. The pellets were dissolved in RNA storage buffer (Ambion, Austin, USA) and stored in −80℃ until used for re-

verse-transcription (RT) reaction. The qualities and quantities of total RNAs were determined by gel electrophoresis and an UV spectrophotometer (Eppendorf, New York, USA), respectively. Generation of the first stranded cDNA was carried out according to the instruction of ImProm-II™ reverse transcription system (Promega, Madison, USA). Briefly, 1 μg of total RNA was used for a RT reaction, in total volume of 20 μl with oligo-dT primer. The RT reaction was carried out at 25 ℃ for 5 min, 42℃ for 1 hr, and 70℃ for 15 min. The cDNA was directly utilized for semi-quantitative real-time PCR.

Procedure of Semi-Quantitative Real-Time PCR Analysis

The semi-quantitative real-time PCR was performed in a mixture of 1 μl of cDNA, 10 pmol of each primer, and 10 μl of master mix (Finnzymes, Espoo, Finland). A total volume of the mixture for semi-quantitative real-time PCR was 20 μl. The employed PCR procedure was an initial step of pre-denaturation at 95 ℃ for 5 min, followed by cycles of denaturation at 95 ℃ for 30 sec, annealing at T_m for 30 sec, and extension at 72℃ for 30 sec. The final extension at 72 ℃ for 10 min was added to make sure all PCR products in double-stranded forms. The information of oligonucleotide primers used for PCR is shown in Table 1. The primers were prepared by either utilizing published information or using Primer 3 software (http: //www.bioneer,co.kr/cgi-bin/primer/primer3.cgi: Whitehead Insti-

Table 1. Summary of primer information and PCR condition for semi-quantitative real-time PCR

Gene (GenBank access number)	Primer sequence	T_m (℃)	Expected PCR size (bps)	Note
Cx26 (NM_001004099)	F : TCCTCTTCATCTTCCGCATC (20~39) R : CCGTTTCTTTTCGTGTCTCC (233~252)	55	233	N.D.
Cx30 (NM_053388)	F : CAATCTCGTGGACTGCTTCA (525~544) R : ATGGCATTCTGACCGCTATG (748~767)	55	243	N.D.
Cx30.3 (NM_053984)	F : CCCAATGTCTGCTATGACGA (181~200) R : CACAGCAGCCTTGAAGATGA (404~423)	57	243	D.
Cx31 (NM_019240)	F : TTGAGCGGTGTGAACCAGTA (28~47) R : TGTTGGAGATGGGGAAGAAG (201~220)	57	193	D.
Cx31.1 (NM_019241)	F : CATCGTCTGCATCCTGCTTA (576~595) R : ATGAGGTCGCTTGAGAGGAA (721~740)	55	165	D.
Cx32 (NM_071251)	F : AGAATCATGGTGCTGGTGGT (94~113) R : CCTCAAGCCGTAGCATTTTC (309~328)	57	235	D.
Cx33 (NM_019308)	F : TGAGAGGCAGATTGCTGCTA (443~462) R : AGACACCATTGACACCACCA (644~663)	57	221	N.D
Cx36 (NM_019281)	F : TCTGGAGATTGGGTTTCTGG (624~643) R : CGGACAGCCAGTTTGATCTT (835~854)	58	231	N.D
Cx37 (M76532)	F : AGTGTCTGTACCTTGGATGCC (1147~1167) R : CAGCACACTTAGCCAAGAGC (1350~1369)	51	223	D.
Cx40 (NM_019280)	F : ATACCATTCAGCCTGGTTGC (164~183) R : CGGCCTCTTTAGCTTTCTCA (333~352)	57	189	D.
Cx43 (NM_012567)	F : AGCAAGCTAGCGAGCAAAAC (908~927) R : GAGTTCATGTCCAGCAGCAA (1040~1059)	55	151	D.
Cx45 (NM_001085381)	F : GATCATCCTGGTTGCTACTC (240~259) R : GATCCTCTTCATGGTCCTCT (412~431)	57	173	D.
Cx50 (NM_0153465)	F : CCACTCCATTGCAGTTTCCT (777~796) R : AGAAGGCAGGGTTTCTTGGT (987~1006)	57	211	N.D.
Ppia (NM_017101)	F : GGCAAATGCTGGACCAAACAC (342~362) R : TTAGAGTTGTCCACAGTCGGAGATG (513~537)	59	196	D

Cx: connexin; *Ppia*: cyclophilin A; N.D.: not detected; D.: detected.
Numbers in parentheses of primer sequences indicate the positions of bases in GenBank sequences.

tute/MIT Center for Genomes Research, USA). The PCR products were confirmed by fractionation on 1.2% agarose gels. In this analysis, we included cyclophilin A (*Ppia*) as an internal PCR control.

Data Analysis and Presentation

A mean and a standard error for each experimental group were obtained from 5 repeated analyses of RT and semi-quantitative real-time PCR. The expression levels of connexin isoforms in the IS were compared with those of *Ppia* and presented as relative ratios between *Cx* and *Ppia*. Statistical mean differences among 7 experimental groups for each connexin isoform were determined by one-way ANOVA, followed by a post-hoc analysis, Duncan's test. In all cases, results were considered significant if $p<0.05$ level.

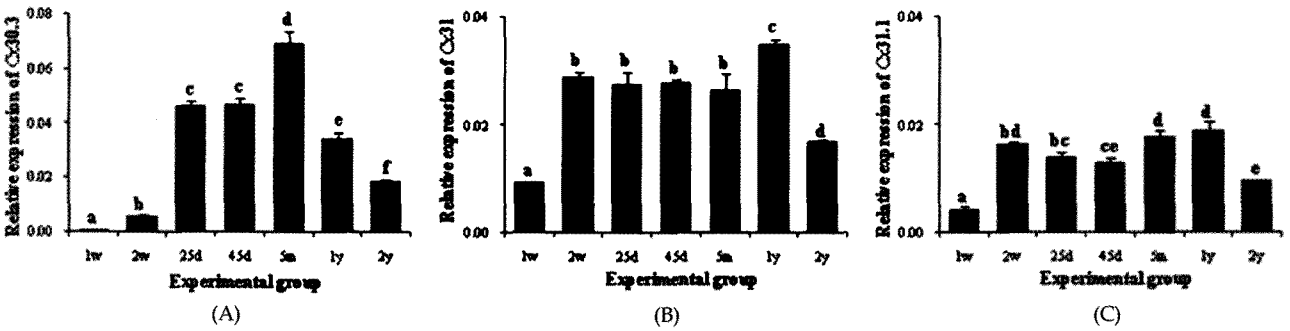
RESULTS

Expression of Connexin 30.3, 31, and 31.1 in the Initial Segment of the Male Reproductive Tract during Postnatal Development

A minimal expression of *Cx*30.3 was detected at 1 week of age (Fig. 1A). However, significant increases of *Cx*30.3 mRNA levels were observed at 2 weeks and 25 days of ages (Fig. 1A). Another increase of *Cx*30.3 mRNA abundance was seen at 5 months of age, followed by a significant decrease at 1 year of age (Fig. 1A). The level of *Cx*30.3 mRNA was further significantly decreased at 2 years of age, compared with that at 1 year of age (Fig. 1A). The mRNA level of *Cx*31 in the IS was significantly increased at 2 weeks of age, compared with that at 1 week of age (Fig. 1B). This level of *Cx*31 mRNA was remained until 5 months of age (Fig. 1B). Interestingly, an increase of *Cx*31 mRNA abundance was detected at 1 year of age, followed by a significant decrease of *Cx*31 mRNA level at 2 years of age (Fig. 1B). The mRNA level of *Cx*31.1 at 2 weeks of age was significantly increased, compared with that at 1 week of age (Fig. 1C). Gradual decreases of *Cx*31.1 mRNA levels were observed at 25 and 45 days of ages, followed by a significant increase at 5 months of age until 1 year of age (Fig. 1C). However, the mRNA abundance of *Cx*31.1 was significantly decreased at 2 years of age (Fig. 1C).

Comparison of Connexin 32, 37, and 40 Gene Expression in the Initial Segment at Different Postnatal Ages

Expression of *Cx*32 mRNA was detected at all experimental age groups (Fig. 2). Compared with the level of *Cx*32 mRNA at 1 week of age, significant increases of *Cx*32 mRNA levels were observed at 2 weeks and 25 days of ages (Fig. 2A). However, the level of *Cx*32 mRNA at 45 days of age was significantly decreased to the level at 2 weeks of age (Fig. 2A). At 5 months and 1 year of ages, the levels of *Cx*32 mRNA were significantly increased, compared with that at 45 days of age, even through the mRNA abundance of *Cx*32 at 2 years of age was significantly lower than that at 1 year of age (Fig. 2A). Expressional pattern of *Cx*37 mRNA during postnatal development is shown in Fig. 2B. Compared with the level of *Cx*37 mRNA at 1 week of age, a significant increase was detected at 2 weeks of age, followed by gradual but significant decreases of *Cx*37 mRNA abundance by ages until 5 months of age (Fig. 2B). The level of *Cx*37 mRNA at 5 months of age was minimal (Fig. 2B). However, a surge of *Cx*37 mRNA level was found at 1 year of age, followed by a transient decrease of *Cx*37 gene expression to the minimal level at 2 years of age (Fig. 2B). Gene expression of *Cx*40 was different with those of *Cx*32 and 37 (Fig. 2C). Tremendous increases of *Cx*40 mRNA levels were observed at 2 weeks and 25 days of ages, compared with that at 1 week of age (Fig. 2C). However, the abundance of *Cx*40 mRNA was remained at the level until 5 months of age, while the mRNA levels of *Cx*40 at 1 and 2 years of ages were signi-



(A) (B) (C)

Fig. 1. Gene expression patterns of connexin 30.3, 31, and 31.1 in the initial segment during postnatal development. Expression of *Cx*30.3 (A), *Cx*31 (B), and *Cx*31.1 (C) mRNA in the IS. 1w: 1 week, 2w: 2 weeks, 25d: 25 days, 45d: 45 days, 5m: 5 months, 1y: 1 year, and 2y: 2 years of age. Means with different letters are significantly different, if $p<0.05$.

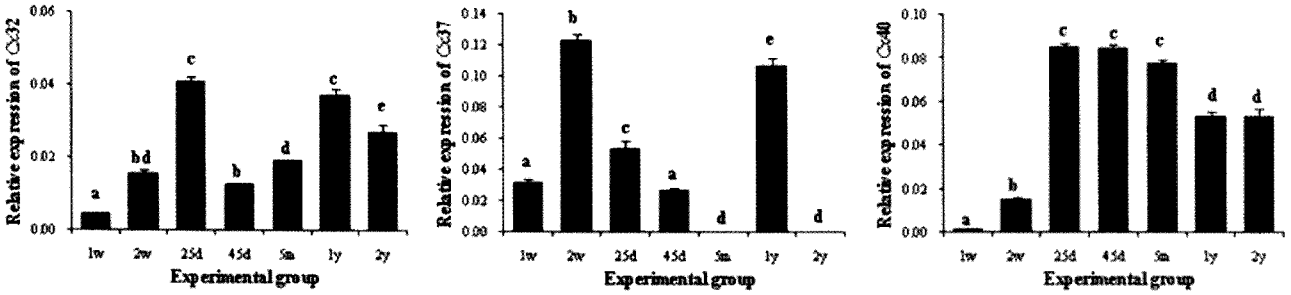


Fig. 2. Expressional patterns of connexin 32, 37, and 40 in the initial segment at different postnatal ages. Expression of *Cx*32 (A), *Cx*37 (B), and *Cx*40 (C) mRNA in the IS. 1w: 1 week, 2w: 2 weeks, 25d: 25 days, 45d: 45 days, 5m: 5 months, 1y: 1 year, and 2y: 2 years of age. Means with different letters are significantly different, if $p<0.05$.

ficantly lower than that at 5 months of age (Fig. 2C).

Expressional Patterns of Connexin 43 and 45 mRNAs in the Initial Segment during Postnatal Development

Expression patterns of *Cx*43 and 45 in the IS during postnatal development are shown to be similar, as presented in Fig. 3. Significant increases of *Cx*43 and 45 mRNA levels were detected at 2 weeks of age, compared with those at 1 week of age (Fig. 3A and B). However, mRNA levels of these molecules at 25 days of age were significantly decreased to the levels at 1 week of age (Fig. 3A and B). Further significant decreases of *Cx*43 and 45 were seen at 45 days of age (Fig. 3A and B). These levels of *Cx*43 mRNA were remained in a consistent level until 2 years of age (Fig. 3A). However, mRNA abundance of *Cx*45 at 5 months of age was significantly lower than that at 45 days of age (Fig. 3B). Another significant increase of *Cx*45 mRNA level was detected at 1 year of age, followed by a significant decrease at 2 years of age (Fig. 3B).

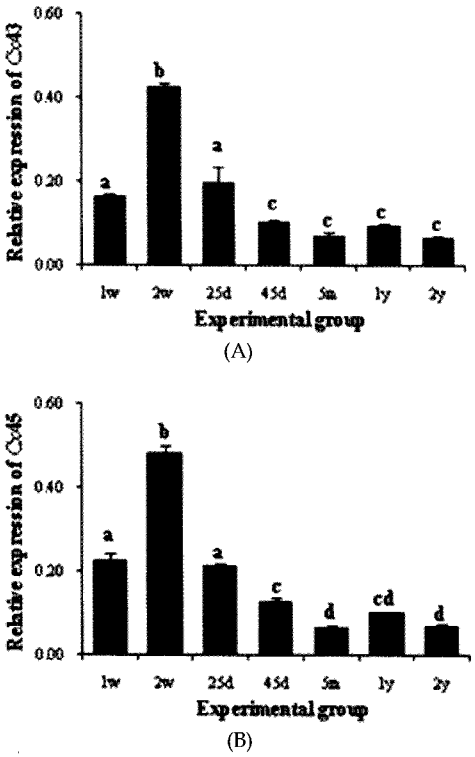


Fig. 3. Comparison of connexin 43 and 45 gene expression in the initial segment during postnatal development. Expression of *Cx*43 (A) and *Cx*45 (B) mRNA in the IS. 1w: 1 week, 2w: 2 weeks, 25d: 25 days, 45d: 45 days, 5m: 5 months, 1y: 1 year, and 2y: 2 years of age. Means with different letters are significantly different, if $p<0.05$.

DISCUSSION

The present study was conducted to examine the presence and expression patterns of a number of *Cx*s in the IS of the male reproductive tract throughout postnatal developmental period. Our results showed that 8 of 13 *Cx*s tested were present in the IS, namely *Cx*30.3, 31, 31.1, 32, 37, 40, 43, and 45. Expression patterns of most *Cx*s during postnatal development were somewhat unique, while a similar expression patterns was observed in *Cx*43 and 45. Expression levels of *Cx*32 and 37 mRNAs during postnatal developmental period were fluctuated. Transcript levels of *Cx*30.3, 31, 31.1, 32, and 40 were elevated at early prepubertal age and generally remained in higher levels at pubertal and adult ages. In contrast, levels of *Cx*43 and 45 mRNAs were significantly decreased at and after postpuberal ages. Thus, these results imply that expression of *Cx*s in the IS during postnatal development would be differentially regulated by a number of factors, including the testicular and/or extratesticular-originated molecule(s).

The epididymis is the part of the male reproductive tract, at which maturation of spermatozoa produced from the testis occurs. As described earlier in the introduction section, the IS has a number of unique histo-

chemical and morphological characters discriminated from the rest of the epididymis. Even though the principal cells are the major cell type of epithelial cells in the IS, the relative percentile contribution of the principal cells is the highest in the IS, compared with those in other epididymal parts (Robaire and Hermo, 1988). In addition, unique cell types, apical and narrow cells, are only found in the IS (Adamali and Hermo, 1996). These cells are distinguished from other cells, in respect of structural and histochemical features (Adamali and Hermo, 1996; Robaire and Hermo, 1988). For example, an intensive immune-reactivity of carbonic anhydrase II is detected in the narrow cells of the IS epithelium (Adamali and Hermo, 1996). Moreover, the luminal composition within the IS is different from those within the rest of the epididymis. The luminal fluid of the IS contains a relatively high concentration of Na^+ and low concentrations of K^+ and PO_4^{2-} (Robaire and Hermo, 1988). In addition, a transient increase of inositol concentration is detected in the IS fluid (Robaire and Hermo, 1988). Together, these evidences clearly inform that the IS plays an important role(s) on sperm maturation, which is distinctive from the rest of the epididymis. A mechanism of establishment of regional specific microenvironment in the epididymal fluid is very complicate and has not been well characterized. However, it is suggested that segmental differences in epididymal physiology would be results of a coordinated function among different cell types in the epididymal epithelium, and thus cell-cell communication appears to be important to epididymal function (Dufresne *et al.*, 2003).

Direct communication between adjacent cells is occurred through gap junction, which forms transmembrane channels allowing the passage of small molecules (Dufresne *et al.*, 2003). In the male reproductive tract, the testis possesses 11 *Cx* isoforms, including *Cx*26, 30.2, 31, 31.1, 32, 33, 37, 40, 43, 46, and 50 (Haefliger *et al.*, 1992; Pointis *et al.*, 2005). In the efferent ductules of the male reproductive tract, expression of several *Cx* isoforms has been demonstrated from our earlier study (Lee *et al.*, 2007). The expression and localization of multiple *Cx* isoforms in the epididymis have been demonstrated by a number of previous researches (Cyr *et al.*, 2002; Dufresne *et al.*, 2003; Pointis *et al.*, 2005). Developmental patterns of *Cx*26, 31.1, 32, and 43 in the epididymis have been examined (Dufresne *et al.*, 2003). Expression patterns of certain *Cx* isoforms in the epididymis are age-dependent, while other *Cx* isoforms are constitutively expressed in the epididymis regardless postnatal development (Dufresne *et al.*, 2003). Moreover, some *Cx* isoforms present in the epididymis shows segmental-specific expression patterns (Dufresne *et al.*, 2003). However, none of researches has specifically determined expression patterns of *Cx* isoforms in the IS. Our present study demonstrates the expression of 8 *Cx* isoforms in the IS, at least. To our knowledge, this is the first time to report the presence of *Cx*31, 37, 40, and 45 in the IS during postnatal development. In addition, our current study demonstrates that expression patterns of some *Cx* isoforms present in the IS are somewhat very different from other *Cx* isoforms. Such differential expression patterns of multiple *Cx* isoforms in the IS indicate complex regulation of gap junctional communication between adjacent cells during postnatal development. It is also postulated that the presence of different types of *Cx* isoforms in the IS, compared with the rest regions of the epididymis, would influence in creation of regional specific micro-environment in luminal fluid.

Expressional regulation of *Cx* genes in the male reproductive tract has been examined by several researches (Cyr *et al.*, 1996; Pointis *et al.*, 2005; St-Pierre *et al.*, 2003). In the testis, expression of *Cx* genes between same and different cell types is subject to endocrine and paracrine regulation. Follicle stimulating hormone stimulates expression of *Cx*43 between Sertoli cells (Lablack *et al.*, 1998). In addition, retinoic acid and vitamin A would participate in the regulation of *Cx*43 gene expression in Sertoli cell (Pointis *et al.*, 2005). Expression of *Cx*43 mRNA between Leydig cells is altered by human chorionic gonadotropin treatment (You *et al.*, 2000). In the epididymis, Cyr *et al.* (1996) have demonstrated androgen-dependent regulation of *Cx*43 gene expression in the IS. Moreover, thyroid hormone influences *Cx*43 expression in the proximal regions of the epididymis, but not in the cauda epididymis (St-Pierre *et al.*, 2003). As an androgen-dependent organ, development and function of the IS are largely controlled by androgen. Thus, it would not be surprising that expression of *Cx* isoforms in the IS are controlled by testis-producing androgen, at least in part. Even though androgen-dependent expression of *Cx*43 mRNA in the IS has already been demonstrated (Cyr *et al.*, 1996), no information is available for expressional regulation of other *Cx* isoforms detected from the present study. Additional efforts should be made to determine detailed mechanisms on transcriptional regulation of *Cx* genes by androgen in the IS. In addition to androgen, the evidence of the presence of estrogen receptors in the IS suggest a possible role of estrogen on gene expression of *Cx* isoforms in the IS (Mowa and Iwanaga, 2001).

In conclusion, the present study clearly demonstrates the expression of multiple *Cx* isoforms in the IS of the male reproductive tract. Different expression patterns of these *Cx* isoforms during postnatal development would relate with normal development of the IS and establishment of adequate micro-environment in the luminal fluid of the IS for sperm maturation.

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