Blackwell Publishing

Asian J Androl 2007; 9 (4): 515–521
DOI: 10.1111/j.1745-7262.2007.00300.x

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·*Review*·

Epididymis-specific lipocalin promoters

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Abstract

Our goal is to decipher which DNA sequences are required for tissue-specific expression of epididymal genes. At least 6 epididymis-specific lipocalin genes are known. These are differently regulated and regionalized in the epididymis. Lipocalin 5 (*Lcn5* or *mE-RABP*) and Lipocalin 8 (*Lcn8* or *mEP17*) are homologous genes belonging to the epididymis-specific lipocalin gene cluster. Both the 5 kb promoter fragment of the *Lcn5* gene and the 5.3 kb promoter fragment of the *Lcn8* gene can direct transgene expression in the epididymis (*Lcn5* to the distal caput and *Lcn8* to the initial segment), indicating that these promoter fragments contain important *cis*-regulatory element(s) for epididymis-specific gene expression. To define further the fragments regulating gene expression, the *Lcn5* promoter was examined in transgenic mice and immortalized epididymal cell lines. After serial deletion, the 1.8 kb promoter fragment of the *Lcn5* gene was sufficient for tissue-specific and region-specific gene expression in transgenic mice. Transient transfection analysis revealed that a transcription factor forkhead box A2 (Foxa2) interacts with androgen receptor and binds to the 100 bp fragment of the *Lcn5* promoter between 1.2 kb and 1.3 kb and that Foxa2 expression inhibits androgen-dependent induction of the *Lcn5* promoter activity. Immunohistochemistry indicated a restricted expression of Foxa2 in the epididymis where endogenous *Lcn5* gene expression is suppressed and that the Foxa2 inhibition of the *Lcn5* promoter is consistent with the lack of expression of Lcn5 in the corpus and cauda. Our approach provides a basic strategy for further analysis of the epididymal lipocalin gene regulation and flexible control of epididymal function. *(Asian J Androl 2007 July; 9: 515–521)*

Keywords: androgen; epididymis; gene cluster; gene evolution; gene regulation; lipocalin; transcription factor

1 Introduction

In mammals, the epididymis is a specialized organ for sperm storage and sperm maturation. Sperm acquire the capacity for motility and fertilizing ability during transit through the epididymis. There are numerous proteins expressed in a region-specific manner in the epididymis. One of the epididymal proteins, epididymal retinoic acid binding protein (E-RABP or Lipocalin [Lcn] 5), belongs to the lipocalin family and displays a distinct gene expression pattern and regulation in the epididymis [1, 2]. To decipher the fundamental mechanism for epididymis-specific gene expression, we have chosen and studied the mouse *Lcn5* promoter as a model. The findings from the study of the epididymis-specific gene promoters should provide immense possibilities to facilitate epididymal research.

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2 Regionalization of gene (protein) expression

If the sperm maturation process relies on the sequential modification of sperm surface, it is reasonable to search for region-specific proteins secreted into the epididymal lumen. In 1980, Brooks and Higgins [3] reported luminal proteins B, C, D and E in the rat epididymis. They demonstrated that proteins B and C are synthesized in the initial segment of the epididymis and that protein D is secreted in the caput and cauda epididymidis by polyacrylamide gel electrophoresis under non-denaturing and denaturing conditions. Two decades later, although approximately 200 region-specific proteins have been identified in the epididymal fluids of different species, very few epididymal proteins are found to be directly involved in epididymal function [4]. Therefore, there is increasing demand for a robust and simple technique to screen epididymal secretory proteins.

Imaging mass spectrometry (IMS) is currently considered a powerful tool for studying the regionalization of protein expression in the epididymis. IMS was developed by Chaurand *et al*. [5, 6], and can be used to map the distribution of targeted compounds in tissue sections. This technique uses 12 μm frozen sections of the epididymis placed on a sample plate. After deposition of matrix with a diameter of less than 1 mm, protein profiling can be obtained above 200 000 mass-to-charge (*m/z*) with high resolution in the range of 2 000 to 70 000 *m/z* from each matrix spot [7]. In the epididymis, more than 400 signals can be obtained, and at least 50 epididymal proteins, both known and unknown, are found to be expressed in a region-specific manner [6]. In the range of 16 000 to 28 000 *m/z*, Chaurand *et al*. [6] demonstrate that several epididymis-specific proteins, such as cysteine-rich secretory protein 1 (Crisp1) and glutathione peroxidase 5 (GPX5), are detected by IMS, corresponding to previous reports using immunohistochemistry (IHC) or *in situ* hybridization (ISH) [6]. For example, IMS detected Lcn5 with the signals at 18 007 *m/z* (short form) and 18 309 *m/z* (long form). These signals were first detected in the mid-caput and were observed with increasing intensity from mid-caput to cauda, indicating that Lcn5 tends to accumulate in the cauda. These results obtained for Lcn5 by IMS correlate well with the localization data previously obtained by ISH [1] and IHC [8]. Lcn5 mRNA expression is observed only in the principal cells of the mid/distal caput (segments II [faint], III, IV and V). Lcn5 immunoreactivity was first detected in the epididymal epithelial cells and the lumen in the mid caput (segment III) and was present in the lumen from distal caput to cauda. Overall, IMS has proven to be a useful discovery tool for studying epididymis, and region-specific protein expression. We have elected to study Lcn5 further to determine the mechanism of epididymis-specific gene expression.

3 Identification of an epididymis-specific lipocalin: Lcn 5

Lcn5 was first identified as proteins B/C in the rat epididymis [3] and then as mouse epididymal protein 10 (MEP10) in that species [8]. Rat Lcn5 was subsequently purified and the cDNA was cloned by cDNA library screening using a DNA probe based on its predicted amino acid sequence [9]. Mouse Lcn5 was also purified and the N-terminal amino acid sequence was obtained. The mouse Lcn5 cDNA was cloned by reverse transcription polymerase chain reaction (RT-PCR) from murine epididymal mRNA using specific primers designed from the murine Lcn5 N-terminal amino acid sequence [1]. Computer analysis of the cDNAs and genomic sequences [10, 11] showed that rat and murine *Lcn5* are orthologous with 75% identity. The genomic organization of murine *Lcn5* shows 7 exons that closely align to the exon patterns of other lipocalin family genes [12]. Both rat and murine *Lcn5* possess the three conserved sequence motifs characteristic of the lipocalin family. To determine rat Lcn5 3-D structure, it was purified and crystallized [13]. Murine *Lcn5* has not been crystallized yet, but its structure was computer-modeled using the amino acid sequence and the X-ray structure of rat Lcn5. The basic structural framework of both rat and murine Lcn5 is a barrel shape, which forms a deep hydrophobic binding pocket for its ligands. This is the common structure of the lipocalin protein fold, indicating that both rat and murine Lcn5 belong to the lipocalin family [1].

Most lipocalins are extracellular proteins secreted into blood or ductal lumen. As mentioned above, the lipocalin family has several characteristics, such as (i) structural signatures defined by conserved G-X-W in exon 2, T-D-Y in exon 4, K/R in exon 5, and cysteine residues in exon 3 and 5 that are involved in the formation of a disulphide bond [14]; (ii) high conservation of a 3-D structure folding by a single eight-stranded β-sheet forming a β-barrel shape [14], (iii) binding ability for hydrophobic ligands, such as retinoids, fatty acid, bilin and hormones [14–16]; and (iv) high sequence and structural conservation during evolution [12, 14, 17].

Our group found that murine *Lcn5* binds *all-trans*, but not *9-cis*, retinoic acid *in vitro* [15]; although the endogenous ligand of Lcn5 is still unknown. Retinoic acid is important for maintaining epididymal epithelium integrity and function [18] and retinoic acid receptor knockout (KO) mice have an abnormal epididymal epi-

thelium [19, 20]. Recently, we established an Lcn5 KO mouse line to study the physiological function of Lcn5 in the epididymis. Although the Lcn5 KO mice were healthy and produced offspring, the cauda epididymidis of Lcn5 KO mice was not as well developed as that of wild-type mice (Suzuki *et al*. 2007, unpublished data). We then hypothesized that the phenotype might be accentuated if the vitamin A loading is reduced in the diet, because *Lcn5* binds retinoic acid *in vitro* and might be involved in retinoid trafficking in the epididymis. Such is the case for cellular type retinol binding proteins (CRBP I and II) because both CRBP I and CRBP II KO mice are normal when they are fed a diet containing a normal amount of vitamin A. However, when the mice are fed a vitamin A deficient (VAD) diet, CRBP I KO mice demonstrate keratinized epithelial cells in the prostate, bladder and epididymis [21], and CRBP II KO mice show increased neonatal lethality [22]. Indeed, we observed a typical VAD effect, like keratinized epithelium, in the Lcn5 KO mice fed a VAD diet. In addition, the Lcn5 KO mice demonstrate epididymitis defined by massive infiltration of leukocytes into the lumen and rupture of epithelium (Suzuki *et al*., unpublished data). Therefore, we conclude that the *in vivo* function of Lcn5 is to transport retinoic acid in the cauda epididymidis and to prevent inflammation in the epididymis.

4 Identification of an epididymal lipocalin gene cluster

The cDNA encoding mouse *Lcn5* was used to screen a mouse 129 strain genomic BAC library [11]. A positive clone that covers the *Lcn5* gene locus was used for the fluorescence ISH study. The *Lcn5* gene was mapped to the proximal region [A3-B] of murine chromosome 2, which is homologous to the human chromosome 9q and 10p [11]. The [A3-B] locus is already known as a lipocalin-rich area, where lipocalin 2 (Lcn2) (also known as NGAL [23], 24p3 [24] or uterocalin [25]), C8gamma and prostaglandin D2 synthase (Pgds) [16] are localized [11, 12]. Analysis of the 5'-flanking region of the *Lcn5* gene revealed a novel gene, termed mEP17 (murine Epididymal Protein of 17 kilodaltons or lipocalin 8 [Lcn8]), homologous to the *Lcn5* gene and localized 1.7 kb upstream from the transcription starting site of the *Lcn5* gene [26]. Exon/intron boundaries of the *Lcn5* and *Lcn8* genes are identical, suggesting that these genes were generated by tandem *in situ* duplication within the proximal region of mouse chromosome 2. Computer analysis using the mouse genome sequence released by Celera Discovery System and the National Center for Biotechnology Information revealed an epididymal lipocalin gene cluster encompassing the *Lcn5* and *Lcn8* genes and 5 new genes termed *Lcn9*, *Lcn10*, *Lcn11*, *Lcn12* and *Lcn13*, showing approximately 50% homology to *Lcn5* [17]. Although *Lcn11* is detected by nested-RT-PCR as being expressed in the epididymis and other tissues, *Lcn9*, *Lcn10*, *Lcn12* and *Lcn13* are specifically expressed in the epididymis.

Interestingly, the epididymis-specific lipocalin cluster is flanked by other members of the lipocalin family, such as *Pgds* and *Lcn2*, which are expressed in other tissues besides the epididymis, whereas the 6 genes of the epididymal cluster are expressed only in the epididymis [27, 28]. These results suggest that the epididymis-specific lipocalin cluster contains a locus control region that enhances lipocalin gene expression only in the epididymis, whereas genes flanking either side of this epididymal gene cluster are expressed in other tissues besides the epididymis. A comprehensive comparison of the mouse and human genome sequences showed that a homologous lipocalin cluster encompassing the *PGDS*, *LCN12*, *C8gamma*, *LCN8*, *LCN5*, *LCN10*, *LCN9* and *LCN2* genes were found on mouse chromosome 2 and human chromosome 9. Hall's group has reported a human Lipocalin 6 (*LCN6*) specifically expressed in the epididymis [29]. This gene is the orthologous gene of mouse *Lcn5*. Taken together, this epididymal lipocalin gene cluster appears to have arisen by tandem *in situ* duplication of an ancestral gene *Pgds* determined by phylogenetic analysis [17]. This high degree of conservation during evolution between mouse and human suggests that the epididymal lipocalins play an important role in epididymal function.

Another feature of the epididymal lipocalin cluster is different region-specific expression patterns. ISH reveals that *Lcn5* is expressed in the mid/distal caput epididymidis, *Lcn8* and *Lcn9* are expressed only in the initial segment, whereas *Lcn10* is expressed not only in the initial segment, but also in the upper margin of the distal caput [1, 17, 26]. *Pgds* has broad gene expression through the corpus and cauda by ISH [27] and *Lcn2* is expressed through the caput and corpus by northern blot analysis [30]. However, by ISH, we have found *Lcn2* gene expression to be restricted to the initial segment, as are *Lcn8* and *Lcn9* (Suzuki *et al*. 2007, unpublished data).

The hormonal regulation of the epididymal lipocalin genes is also different. *Lcn5* is androgen-dependent, *Lcn8*, *Lcn9* and *Lcn13* are testicular factor-dependent, and *Lcn10* and *Lcn2* are both androgen-dependent and testicular factor-dependent [17]. According to these results, the epididymis-specific lipocalin gene cluster displays different epididymal spatial expression and different gene regulation.

5 Identification of DNA sequences required for tissue-specific expression of epididymal lipocalin genes in the *Lcn5* promoter

Although the *Lcn5* gene expression is tightly restricted in the mid/distal caput epididymidis, little is known about how this mechanism is controlled. To determine which DNA sequences are required for tissue-specific expression of epididymal genes, both the *Lcn5* and *Lcn8* gene promoters were studied. The 5 kb 5'-flanking region of the *Lcn5* gene directs the transgene expression to the mid/distal caput epididymidis of transgenic mice in a manner that mimics endogenous *Lcn5* gene expression [31]. This transgenic mice study indicates that the 5 kb fragment, but not the 0.6 kb, contains all the information required for the mid/distal caput-specific gene expression. We have narrowed down the 5 kb fragment to 3.8 kb and 1.8 kb fragments in transgenic mice. Both constructs still drive mid/distal caput-specific gene expression, indicating that the 1.8 kb fragment of the *Lcn5* promoter is essential for the epididymis- and mid/distal caput-specific gene expression [32, 33]. To narrow down the 1.8 kb *Lcn5* gene promoter fragment further, we needed appropriate epididymal cell lines because cells from other tissues might not reflect the tissue-specific gene regulation observed in normal epididymal cells *in vivo*. Therefore, we established immortalized epididymal cell lines from a transgenic mouse carrying temperature-sensitive (ts) SV40 large T antigen [34]. At body temperature, the ts SV40 large T antigen will be degraded. However, when the cells from the transgenic mouse are isolated and cultured *in vitro* at 33ºC, the cells express the functional large T antigen, which induces immortalization of the cells. When the cells are cultured at 39ºC, T antigen is degraded and the cells can differentiate [35]. The epididymis was isolated and separated into proximal caput and distal caput region and the isolated cells were cultured at 33ºC. Using serial cup cloning procedures, we established four monoclonal immortalized cell lines; one from the proximal caput region (PC1) and three from the distal caput (DC1, DC2 and DC3).

We used DC2 cell line expressing *Lcn5* at the protein and RT-PCR levels for the *Lcn5* promoter assays. To identify the important *cis*-DNA regulatory elements for epididymis-specific gene expression, serial deletion constructs of the 1.8 kb fragment of the *Lcn5* promoter were ligated to the luciferase gene and transfected into the epididymal DC2 cells [32, 33]. The androgen response was the highest in the 1.3 kb construct, but was lower in the 1.4–1.8 kb fragments, suggesting that there are inhibitory elements between 1.3 and 1.8 kb. Gel shift assay analysis revealed two androgen receptor (AR) binding sites between 1.2 and 1.3 kb, which is consistent the high androgen response of the 1.3 kb construct.

We identified a putative forkhead box A (Foxa) protein binding site close to the two AR binding sites in the 1.3 kb fragment of the Lcn5 promoter (Figure 1A [32]). Foxa proteins (also known as hepatocyte nuclear factor-3 proteins) consist of three subtypes: Foxa1, Foxa2 and Foxa3. They belong to a large forkhead box transcription family [36]. The putative mechanism of Foxa1 transcriptional regulation is to displace histones and to open up chromatin structures to disassemble nucleosome [37, 38]. These Foxa proteins are expressed in the endoderm and are important during embryo development [39, 40]. In adult mice, Foxa1 is strongly expressed in liver, and also detected in prostate, seminal vesicle and bladder [41, 42]. Interestingly Foxa1 regulates prostate-specific genes, such as prostate specific antigen (PSA) and probasin [43, 44]. Our work on these prostate-specific genes has identified that these prostate-specific promoters require the Foxa binding sites to be close to the AR binding sites for prostate-specific gene expression [43]. Indeed, AR binding sites and Foxa binding sites are closely localized in the PSA and probasin promoters. Chromatin immunoprecipitation (ChIP) and glutathione-S-transferase (GST)-pull down assay revealed that Foxa1 constantly occupies the PSA promoter DNA fragment in the absence or presence of dihydrotestosterone *in vivo* and that Foxa1 and AR can directly interact. Therefore, we concluded that Foxa1 and AR interaction is important for prostate-specific gene expression.

However, the putative Foxa binding site identified in the Lcn5 promoter is for Foxa2, not Foxa1. We confirmed that Foxa1 is expressed in the prostate but Foxa2 is expressed in the epididymis by RT-PCR and IHC (Figure 1B) [32]. Foxa1 is only detected in the nuclei of the prostatic epithelial cells, whereas Foxa2 is expressed in the nuclei of the epididymal principal cells. By gel-shift assay using nuclear extract from epididymal DC2 cells, we again confirmed Foxa2 binding to the *Lcn5* promoter fragment and interaction of Foxa1 and AR by ChIP and GST-pull down assay [32]. When Foxa2 is over-expressed in the cultured cells, the androgen response of the *Lcn5* promoter is suppressed in the epididymal cells, whereas the PSA promoter activity in the prostatic cells is activated in the absence of androgen. These results imply that Foxa2 binds to both epididymis-specific and prostate-specific gene promoters but regulates them by different mechanisms. Our group has recently found that Foxa2 is strongly expressed in advanced androgen-independent prostate cancer [45]. Foxa2 is only detected in neuron-endocrine small cell carcinoma, which possesses androgen independence and high metastasis ability.

Although the role of Foxa2 in prostate cancer development is not clear, Foxa2 might be involved in the transforming of hormonal regulation into an androgen-independent manner in prostate cancer.

6 Use of epididymis-specific promoter for epididymal research

As of today, several epididymal promoters, including Cres, Pem, Gpx-5 and Crisp1, have been examined to study which *cis*-DNA regulatory elements are important for epididymal gene regulation [46–48]. At least two epididymal promoters, *Lcn5* and *Lcn8*, are proven to be epididymis-specific [31, 49]. These promoters can be used for two purposes. First, as described above, promoters can be used to identify *cis*-DNA regulatory elements to investigate molecular mechanisms that might lead identification of a key molecule important for epididymal function. Second, the ability of the epididymal promoters to drive transgene expression in the principal cells in specific regions of the epididymis should be useful for generating transgenic mice. A Cre-loxP system allows us to disrupt specific gene function in a specific region in the epididymis. Because numerous loxP-floxed

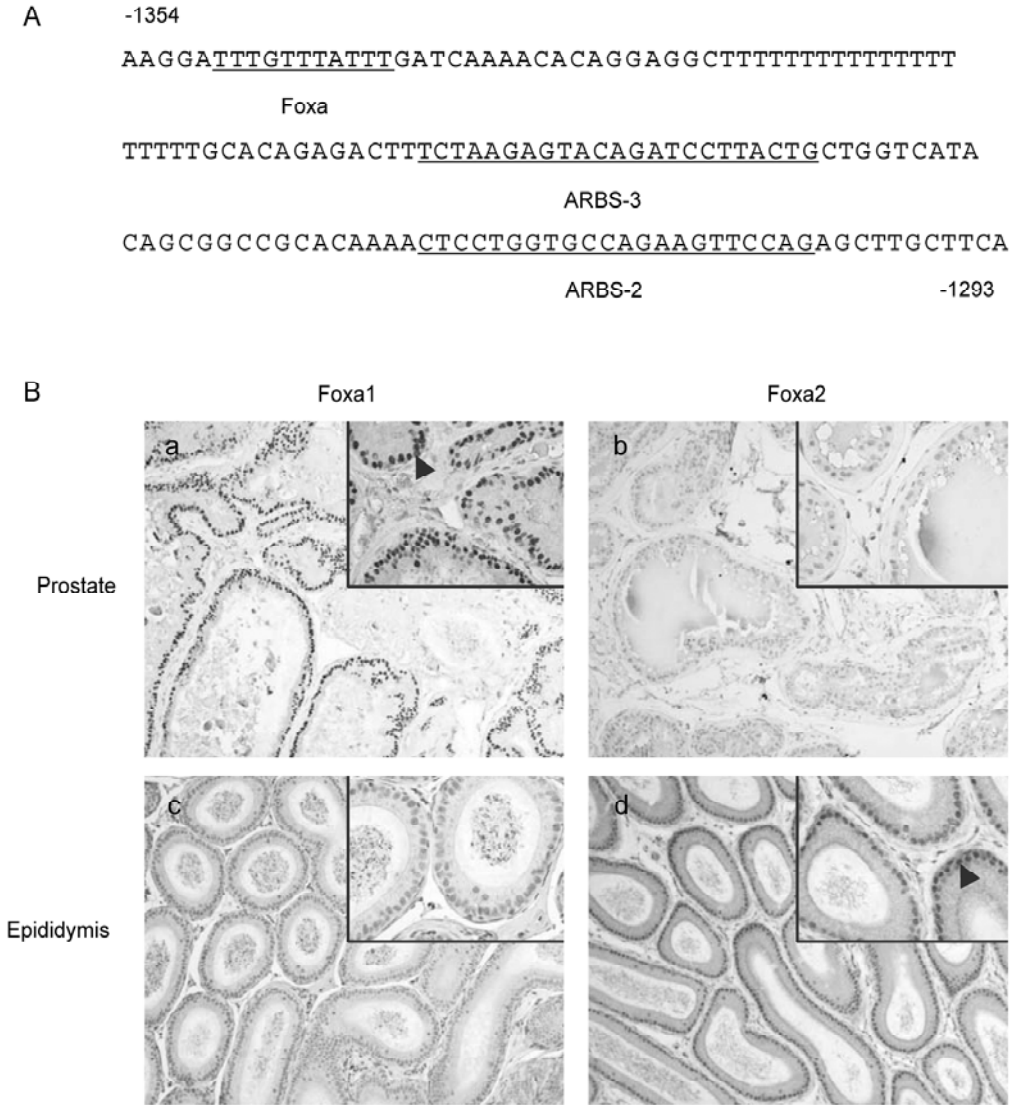

Figure 1. (A): Forkhead box A2 (Foxa2) binding site on the Lipocalin 5 (Lcn5) promoter from -1354 to -1293 bp. Underlined sequences are the putative binding site for Foxa and androgen receptor binding sites (ARBS). (B): Immunohistochemistry analysis of Foxa proteins in the prostate and the epididymis using anti-Foxa1 antibody (a and c) and anti-Foxa2 antibody (b and d). Both closed triangles indicate strong staining of nucleus. Low magnification: 100 ×; high magnification: 400 ×. Modified with permission from Yu *et al*. [32].

gene KO mice are available, crossing with transgenic mice expressing Cre recombinase under the control of *Lcn8* or *Lcn5* promoter should generate epididymis-specific gene inactivation, which might interfere with the sperm maturation process. The information gained from these conditional KO mice might provide a new aspect in the understanding of epididymal physiology.

7 Conclusion

Here, we reviewed our group's research on the isolation of epididymis-specific proteins that display region-specific patterns. *Lcn5* and *Lcn8* are currently focused on and intensively studied for gene regulation mechanisms. Foxa2 is a co-regulator interacting with AR for epididymis-specific gene expression of *Lcn5*. There must be other factors that remain unknown in the epididymis. If one could determine key molecules that regulate epididymis-specific genes, such molecules would be ideal targets for developing synthesized compounds for male contraceptive drugs. In addition, these epididymis-specific promoters would be useful for generating epididymis-specific Cre-expressing transgenic mice, which help to disrupt genes of interest in epididymis-specific and region-specific patterns. Therefore, epididymal promoter research is leading to the accumulation of knowledge that will accelerate the regulation of epididymal function and help to uncover new classes of drugs.

Acknowledgment

This research was supported by the Ernst Schering Research Foundation (Germany), the Rockefeller Foundation (USA), Contraceptive Research and Development Program (USA) and the National Institutes of Health HD36900 and DK55748 (USA).

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