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Segment - and Cell- Specific Expression of D-type Cyclins in the Postnatal Mouse Epididymis

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Abstract

Sperm transport, maturation and storage are the essential functions of the epididymis. The epididymis in the mouse is structurally characterized by regional and segmental organization including caput, corpus and cauda epididymis that are comprised of ten segments. Although several growth factor signaling pathways have been discovered in the epididymis, how these converge onto the cell cycle components is unknown. To begin to elucidate the growth factor control of cell cycle events in the epididymis, we analyzed the expression of D-type cyclins at different postnatal ages. At 7d, cyclin D1 was mainly expressed in the cauda epithelium, by 14d its expression occurred in the epithelium of caput, corpus and cauda that persisted up to 21d. By 42d, cyclin D1 was mostly detectable in the principal cells of the caput and corpus (segments 1–7) but not in the cauda epididymis. Expression of cyclin D2, unlike that of cyclin D1, was evident only at 42d but not earlier, and was mostly confined to corpus and cauda epithelium. In contrast to both cyclin D1 and D2, cyclin D3 was expressed primarily in the interstitium at 7d and by 21d its expression was localized to the epithelium of the corpus and cauda epididymis. By 42d, expression of cyclin D3 peaked in segments 6–10 and confined to basal and principal cells of the corpus and apical cells of the cauda epithelium. Ki67 immunoreactivity confirmed absence of cell proliferation despite continued expression of D-type cyclins in the adult epididymis. Collectively, on the basis of our immunophenotyping and protein expression data, we conclude that the D-type cyclins are expressed in a development -, segment-, and cell- specific manner in the postnatal mouse epididymis.

Keywords

Epididymis; Cyclin D1; Cyclin D2; Cyclin D3; Ki67

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1. Introduction

The epididymis is an important male accessory sex organ that plays key roles in sperm transport, maturation and storage (Cooper and Yeung, 2010; Cornwall, 2003; Hess, 2002; Robaire, 2005). It is derived from the Wolffian duct during embryonic male reproductive tract development and differentiates under the influence of testosterone (Cornwall, 2003; Robaire, 2005; Rodriguez et al., 2002). Ultrastructural analyses of the epididymis (Ilio and Hess, 1994; Robaire, 2005), and effects of various toxicants on epididymal sperm structure and maturation have all been extensively studied (Howdeshell et al., 2008; Marty et al., 2003). Anatomically, the epididymis is organized into three regions including caput, corpus and cauda epididymis that are further sub-divided into various segments (Abe et al., 1984; Adamali et al., 1999; Hess, 2002; Robaire, 2005). In the mouse, the epididymis consists of 10 distinct segments, whereas that of the rat is comprised of 14 segments (Adamali et al., 1999; Hess, 2002; Robaire, 2005). The epididymis predominantly consists of an epithelial compartment surrounded by myoid cells and a stromal compartment (Cornwall, 2003; Hess, 2002; Robaire, 2005). Marked cell differentiation within the postnatal epididymis does not occur until after puberty coincident with a rise in circulating androgen levels (Cooper and Yeung, 2010; Ezer and Robaire, 2002; Robaire, 2005).

The epithelial compartment is composed of distinct cell types along the three major regions of the epididymis. The principal cell is the main cell type and comprises approximately 65 % of the total epithelial cell population in the epididymis (Robaire, 2005; Trasler et al., 1988). Active protein secretion and presence of endocytosis machinery are characteristics of this cell (Dacheux and Dacheux, 2002; Robaire, 2005). Apical and narrow cells are positioned adjacent to the principal cells in the epithelium, mostly in the initial segment and intermediate zone. These two types of cells are characterized by expression of proteolytic and lysosomal enzymes (Cornwall, 2003; Cyr et al., 2002; Hermo and Robaire, 2002; Robaire, 2005). Clear cells are another important type of cells present in the epithelium of caput, corpus and cauda. These are large endocytic cells and play a major role in acidification of the luminal fluid (Cornwall, 2003; Cyr et al., 2002; Hermo and Robaire, 2002; Robaire, 2005). Basal cells represent a unique cell type in the epithelium of the epididymis. These cells adhere to the basement membrane and it was believed that they do not have a direct access to the lumen of the duct (Hermo et al., 1994; Hermo and Robaire, 2002; Hess, 2002; Robaire, 2005). However, a recent study using three dimensional confocal imaging has identified that the epididymal basal cells extend long cytoplasmic projections that reach toward the lumen and thus are postulated to scan and chemically sense the luminal environment (Shum et al., 2008). Various functions have been attributed to basal cells including active protein secretion, endocytosis, a role in cell-mediated immune recognition and regulation of electrolyte and water transport (Cyr et al., 2002; Hermo and Robaire, 2002; Hess, 2002; Robaire, 2005; Wong et al., 2002).

The important roles of several growth factor signaling pathways that are critical to growth and differentiation of the epididymis have been well studied (Robaire, 2005; Tomsig and Turner, 2006; Turner et al., 2007b). Furthermore, genetic studies using mutant mouse models have identified defects in morphogenesis (Bomgardner et al., 2003; Kitagaki et al., 2011; Podlasek et al., 1999), development (Robaire, 2005; Tomaszewski et al., 2007;

Yoshio et al., 2010), and function (Grover et al., 2005; Krishnamurthy et al., 2001; Krishnamurthy et al., 2000; Ma et al., 2004; Mendive et al., 2006) of the epididymis as a result of deletions engineered in various gene loci.

The D-type cyclins, cyclin D1, D2 and D3 are critical regulators of the G1-S phase transition of cell cycle (Coudreuse and Nurse, 2010; Hengstschlager et al., 1999; Ikushima and Miyazono, 2010; Malumbres and Barbacid, 2009; Molinari, 2000; Musgrove et al., 2011; Nakayama et al., 2001; Sherr, 2000; Turner and Grose, 2010). These proteins are developmentally regulated by various growth factors in many tissues (Coudreuse and Nurse, 2010; Deshpande et al., 2005; Ikushima and Miyazono, 2010; Malumbres and Barbacid, 2009; Musgrove, 2006; Musgrove et al., 2011; Stein et al., 2006; Turner and Grose, 2010). Although the pattern of expression of D-cyclins is distinct in different tissues, functional redundancy exists among them (Ciemerych et al., 2002; Ciemerych and Sicinski, 2005; Deshpande et al., 2005). The cyclin proteins physically associate and form complexes with cyclin-dependent kinases and phosphorylate cell cycle inhibitors such as p27 kip (Borriello et al., 2007) and the retinoblastoma protein (Bloom and Cross, 2007; Hengstschlager et al., 1999). Interactions among D-type cyclins, and cell cycle inhibitors specify proliferation or differentiation fates of many cells (Coudreuse and Nurse, 2010; Ikushima and Miyazono, 2010; Malumbres and Barbacid, 2009; Musgrove et al., 2011; Turner and Grose, 2010).

Recent studies have identified dynamic gene expression patterns along the mouse, rat and human epididymal segments and many genes including cell cycle regulators show segment specific expression pattern (Dube et al., 2007; Jelinsky et al., 2007; Johnston et al., 2005; Turner et al., 2007a). However, it is unknown if similar expression patterns occur in parallel in the corresponding proteins encoded by these genes in various segments. Although expression of the D-type cyclin proteins has been analyzed in several tissues (Beumer et al., 2000; Carthon et al., 2005; Ciemerych et al., 2002; Kaplan et al., 2005; Kushner et al., 2005; Ravnik et al., 1995; Zhang et al., 1999), such studies in the epididymis are lacking. Moreover, segmental and cell specific expression of several growth factors and receptor signaling systems have also been studied in epididymis (Henderson et al., 2006; Hess et al., 2011; Kitagaki et al., 2011; Lei et al., 2003; Robaire, 2005; Sipila et al., 2011; Tomaszewski et al., 2007; Tomsig and Turner, 2006; Turner et al., 2007b; Yoshio et al., 2010; Zhao et al., 2001). However, expression of the downstream cell cycle components including cyclins has not yet been determined. In this study, we focused on D-type cyclins that are key components of the core cell cycle machinery. D-type cyclins integrate growth factor signals and cell proliferation by playing critical roles in G1-S phase of the cell cycle. We used western blot analyses and immunochemistry using specific antibodies to cyclin D1, D2 and D3 and analyzed expression of D-type cyclins in the postnatal mouse epididymis. Here, we report data on development-, segment-, and cell- specific expression of D-type cyclins in the postnatal mouse epididymis. We found novel expression of D-type cyclins strikingly persistent at a time when cell proliferation has completely ceased in the adult mouse epididymis.

2. Results

2.1. Expression of D-type cyclins in the postnatal epididymis by western blot analysis

To determine the expression of cyclins D1, D2 and D3 in the whole epididymis as a function of age, we first performed western blot analyses using protein lysates prepared from mice at days 7, 14, 21 and 42 (Fig. 1A) representing proliferative (7d and 14d), pubertal (21d) and post-pubertal (42d) stages of development. Additionally, we also analyzed expression of D-type cyclins in caput, corpus and cauda regions isolated from the epididymis of mice at postnatal day 42 (Fig. 1B). Two closely migrating bands, one more strongly immunoreactive than the other, were labeled with the cyclin D1 antiserum. Expression of cyclin D1 was significantly higher at 7d compared with levels at 14d and 21d, but was comparable to those at 42d (Fig. 1A). At 42d, expression of cyclin D1 was the highest in the caput region compared with that in corpus and cauda (Fig. 1B). In contrast, expression of cyclin D2 increased with age and the highest levels were found at 42d, predominantly in the cauda region (Fig. 1). Unlike the expression of both cyclin D1 and D2, that of cyclin D3 gradually showed a trend towards decreased expression with age with the lowest levels observed at 42d (Fig. 1A). At this age, cyclin D3 expression was nearly identical in caput and corpus but showed a trend towards increased expression in cauda region of the epididymis (Fig. 1B and D). Together, these results indicate that cyclin D1, D2 and D3 proteins are expressed in a distinct development pattern and suggest region-wide trends in expression in the postnatal epididymis.

2.2. Expression pattern of D-type cyclins in the pre-pubertal epididymis

To further characterize the cell- and region- specific expression pattern of D type cyclins in the epididymis of pre-pubertal mice, we have performed immunohistochemistry and analyzed their expression at 7d, 14d, and 21d. At 7d, expression of cyclin D1 was mainly localized to the epithelium of cauda but only few cells in the epithelium of caput and corpus epididymis were immunostained (Fig. 2 a–c). Expression of cyclin D1 was also observed in stromal cells of only the cauda but not caput or corpus epididymis (Fig. 2 a–c). Expression of cyclin D1 was found high in epithelium of all three regions of the epididymis by 14d and remained persistent until 21d (Fig. 2 d–i).

In many tissues, both cyclin D1 and D2 exhibit overlapping patterns of expression and in some cases cyclin D2 can functionally replace cyclin D1 (Carthon et al., 2005; Kushner et al., 2005; Sherr and Roberts, 2004). But in the epididymis, distinct expression pattern of cyclin D2 from cyclin D1 was observed. In contrast to that of cyclin D1, expression of cyclin D2 was very low in epithelium and undetectable in stromal compartment of caput, corpus and cauda regions until 21d (Fig. 3). At these pre-pubertal ages, expression of cyclin D2 was mostly localized to nuclei of apical cells within the cauda epididymis (Fig. 3 c and f). Weak staining of cytoplasm in principal cell also can be detected (Fig. 3 d–i).

Cyclin D3 is usually not expressed in same cells that express cyclin D1 and D2 (Carthon et al., 2005; Kushner et al., 2005; Sherr and Roberts, 2004; Zhang et al., 1999). To determine if expression of cyclin D3 is different compared with cyclins D1 and D2, we next examined cyclin D3-immunostained sections of the epididymis from mice at 7d, 14d and 21d. As

predicted, the expression of cyclin D3 was predominantly confined to the interstitial stromal compartment, unlike that of cyclins D1 and D2 at 7d (Fig. 4 a–c). But interestingly, cyclin D3 levels gradually increased in the epithelium of corpus and cauda and they were undetectable in the interstitium by 21d (Fig. 4 d–i).

2.3. Segment-, cell- specific expression of D-type cyclins in the adult epididymis

In order to further clarify the expression pattern of D-type cyclins in the adult epididymis, we have examined their expression in all segments of the epididymis at 42d. Results demonstrated that cyclin D1 was expressed mostly in caput and corpus (segments 1–7; with the highest expression confined to segments 5–7), but only low levels in the cauda epididymis (Fig. 5B). Expression of cyclin D1 was noticeably localized to the principal, but not basal or apical cells of the caput epididymis (Fig. 6 a, d, g, j and m). Thus, the above data suggest there is an anterior-posterior expression gradient of cyclin D1 in the adult epididymis, with maximal levels in the caput and low levels of expression in the cauda region (Fig. 5 B and C).

Differently from cyclin D1, Cyclin D2 was markedly expressed in the corpus and cauda epithelium (Fig. 5B) and was observed both in the cytoplasm and nuclei of principal cells (Fig. 6e, h, k and n). Compared with expression in corpus and cauda, low level expression of cyclin D2 can be observed both in principal and apical cells of caput epididymis (Fig. 6b). Collectively, these results suggest a distinct and an opposite expression pattern of cyclin D2 compared with that of cyclin D1, with minimal expression in the caput and maximal expression in the corpus and cauda epididymis (Fig. 5 B and C).

Similar to the expression of cyclin D2, the expression of cyclin D3 peaked in segments 6–10 of corpus and cauda epididymis (Fig. 5B), but high level of cyclin D3 was detected in initial segment of the epididymis, in the nuclei of principal and apical cells (Fig. 6c). This is in contrast to cyclin D3 expression in corpus and cauda where the distribution of cyclin D3 is confined to cytoplasm of principal cells (Fig. 6f, i, l, o). These observations confirm that there is a development- and cell-type switch in expression of cyclin D3, predominantly from an interstitial stromal to epithelial compartment during early pre-pubertal stages. In addition, different subcellular localization of cyclin D3 between initial/caput and corpus/cauda also imply distinct roles in the differentiation of the epididymis.

2.4. Cell compartment-specific expression of D-type cyclins in segments within the corpus and cauda epididymis of the adult mouse

While mostly non-overlapping pattern of expression of D-type cyclins was observed in the pre-pubertal epididymis, we found co-expression of cyclin D proteins in the corpus and cauda regions in the adult epididymis. To further identify whether they are restricted to distinct cell compartments, we performed immunofluorescence analysis using antibodies to two cyclin D proteins simultaneously (Fig. 7). Although cyclins D1 and D3 were coexpressed in segments, S6 (corpus) and S8 (caput) (Fig. 7 a–f), whereas cyclin D1 was predominantly restricted to the nucleus (Fig. 7 a and c; d and f), and cyclin D3 mainly localized to the cytoplasm (Fig. 7 b and c; e and f). In contrast, cyclin D2 was expressed both in cytoplasm and nucleus in these segments (Fig. 7 g and i; j and l). Interestingly,

within these segments, clear cells did not express cyclins, D1 and D3, but cyclin D2 was detectable (Fig. 7, arrows in d–f and j–l). These data confirm that even when two D-type cyclins are co-expressed in a given cell type within the epididymal segments, their expression is restricted to distinct cellular compartments.

2.5. Discordant expression of cell proliferation marker Ki67 and D-type cyclins

Ki67 is a reliable marker for defining the proliferation status of cells (Neves et al., 2009; Penault-Llorca et al., 2009; Takase et al., 2009). To verify if Ki67 expression was comparable to the expression of D-type cyclins, we evaluated its expression in different regions of the epididymis beginning at 7d and up to 42d (Fig. 8). At 7d, high expression of Ki67 was observed both in epithelium and interstitial cells. An identical expression pattern was observed in the caput, corpus and cauda of epididymis (Fig. 8). Although identical distribution of Ki67 positive cell was observed at 14d, the number of Ki67 positive cells dramatically decreased (Fig. 8). Ki67 expression was detected only in few rare cells by 21d and 42d, (Fig. 8). Thus, despite cell proliferation ceased by 42d, expression of D-type cyclins persisted in the mouse epididymis.

3. Discussion

In this manuscript, we analyzed expression of the D-type cyclins, cyclin D1, D2 and D3 during postnatal development of the epididymis by western blot analyses and immunochemistry. Our data indicate that these proteins are expressed in a spatiotemporally exclusive pattern that is nearly parallel to that reported for the corresponding mRNAs using a large-scale expression profiling method and deposited at <http://mrg.gs.washington.edu/cgi-bin> (Johnston et al., 2005).

An exclusive spatio-temporal and a minimal overlap of expression among the three D-type cyclins has also been observed in other tissues including the testis (Ravnik et al., 1995; Zhang et al., 1999). Similar to the pre-pubertal expression in the epididymis, cyclin D3 expression is also mostly confined to the interstitium (Leydig cells) in the testis (Zhang et al., 1999). Although there is a minimal overlap in expression of the three D-type cyclins, genetic studies with mice lacking individual D-type cyclins do not demonstrate any epididymal defects (Ciemerych et al., 2002; Sicinski et al., 1996; Sicinski et al., 1995; Sicinski and Weinberg, 1997). This lack of an epididymal phenotype in these gene deletion mice could be attributed to two reasons. First, expression of the other two or only one D-type cyclin could go up as a compensatory cell response. Second, since multiple growth factor signaling pathways exist in parallel in the epididymis, it is possible that absence of one D-type cyclin causes a switch in utilization of another D-type cyclin downstream of a signaling pathway.

Comparison of developmental- and segmental-specific expression of D-type cyclins with that of other growth factors, steroid hormone receptors and key cell cycle encoding genes revealed both similarities and differences. For example, bone morphogenetic protein-7, a critical growth factor encoding RNA is expressed along the entire length of the epididymis in pre-pubertal mice, but the expression is more similar to that of cyclin D3, restricted to initial and distal caudal segments in 4 weeks-old mice (Chen et al., 1999). Comparison with

a more recent study shows that *Dusp6* mRNA encoding a phosphatase that preferentially acts on mitogen-activated protein kinase 1/3 is also developmentally regulated in a region-specific manner. It is expressed more abundantly similar to that of *Ccnd2* and *Ccnd3* in the corpus and cauda of the mouse epididymis (Xu et al., 2010).

Segmental-specific expression of mRNA for insulin-like growth factor (*Igf*) 2 resembles that of cyclin D2 - encoding mRNA with increasing levels of expression from proximal to distal segments (<http://mrg.genetics.washington.edu>). In contrast, its cognate receptor *Igfr2* is expressed quite dissimilar to that genes encoding D-type cyclins and is confined to only segments 3–6 (<http://mrg.genetics.washington.edu>). Interestingly, genes encoding fibroblast growth factor (*Fgf*) -8 and 10 are highly expressed in segment 7 unlike that encoding the D-type cyclins and *Fgfr2* mRNA encoding the receptor-2 isoform is expressed only in the cauda (<http://mrg.genetics.washington.edu>). Genes-encoding transforming growth factor superfamily- β 1 and 3 are expressed similar to *Ccnd1* and *Ccnd3* respectively (<http://mrg.genetics.washington.edu>). Signals mediated by steroid hormone receptors regulate epididymal development and function (Bilinska et al., 2006; Brooks, 1983; Hess et al., 2011). Expression analysis of estrogen receptor-1 mRNA (*Esr1*) in the epididymis indicates a pattern similar to that of *Ccnd2*, whereas ESR1 protein expression is restricted to mainly the corpus epididymis by immunochemistry (Hess, 2002; Hess et al., 2011). Androgen receptor mRNA is mainly localized to the initial and caput segments similar to that of *Ccnd1*, whereas androgen co-activator encoding *Ncor1* and *Rnf4* genes are expressed similar to those of *Ccnd1* and *Ccnd3* respectively (<http://mrg.genetics.washington.edu>).

Many key cell cycle protein-encoding mRNAs also demonstrate a segment-specific expression pattern. Cyclin A1-encoding gene is expressed in caput and cauda regions like *Ccnd3* and Cyclin E1-encoding mRNA expression increases across the caput-cauda segments similar to *Ccnd2* (<http://mrg.genetics.washington.edu>). Expression analysis confirms that cyclin-dependent kinase 4-encoding exhibits a pattern similar to those of *Ccnd1* and *Ccnd3* (<http://mrg.genetics.washington.edu>). Expression pattern of genes-encoding cell cycle inhibitors p21 (*Cdkn1a*) and p27 (*Cdkn1b*) are similar to that of *Ccnd2* (<http://mrg.genetics.washington.edu>). Although gene expression database readily provides epididymal segment-specific expression data by microarray analysis, recently proteomic profiling of epididymis segments has also been reported (Chaurand et al., 2003; Guyonnet et al., 2011; Sipila et al., 2009; Suryawanshi et al., 2011). Future studies may provide better correlations to gene-protein expression data sets in the mouse epididymis.

Mice with combinations of mutations that disrupt expression of two D-type cyclins simultaneously and resulting in the expression of only one D-type cyclin have also been generated (Ciemerych et al., 2002; Ciemerych and Sicinski, 2005). Of these double mutants (D1/D2 KO, D1/D3 KO and D2/D3 KO), only a fraction of mice expressing cyclin D3 (D1/D2 KO) survive up to adulthood, but male reproductive tract phenotypes of these mice are unknown (Ciemerych et al., 2002; Ciemerych and Sicinski, 2005). The other double mutants die either during late embryogenesis or within 24–48 h after birth (Ciemerych et al., 2002; Ciemerych and Sicinski, 2005). Development of conditional knockout of D-type cyclins in the epididymis can circumvent this problem in the future.

On the basis of expression pattern of cyclin D1 and cyclin D3 along with Ki67 at pre-puberty, we infer that cyclin D1 is mainly involved in the proliferation of epithelial cells, whereas cyclin D3 is associated with the interstitial cell divisions. We found that D-type cyclins were strongly expressed either in nuclear or cytoplasm of epithelial cells in selected segments at postnatal 42d or beyond (data not shown) when Ki67 expression is rarely detected in all segments of the epididymis. This sustained expression of D-type cyclins in the differentiated adult epididymis could be attributed to two possibilities. First, exclusion of D-type cyclins from nucleus could be a mechanism for stopping cell proliferation. Second, persistent expression of D-type cyclins in post-mitotic cells may be explained by their participation in non-cell cycle mediated processes. This hypothesis is consistent with data that support cell cycle-independent transcriptional up-regulation and functions of D-type cyclins in various cellular contexts (Amanatullah et al., 2002; Coqueret, 2002, 2003; Dey and Li, 2000; Dey et al., 2000; Fu et al., 2004; Jian et al., 2005; Joyce et al., 2001; Kenney and Rowitch, 2000; Pestell et al., 1999; Wang et al., 2004). More recently, a combined genetic-proteomic analysis identified several non-cell cycle proteins that interact with cyclin D1 (Bienvenu et al., 2010). In vivo chromatin immunoprecipitation analyses also revealed occupancy of cyclin D1 on promoters of various genes (Bienvenu et al., 2010).

In conclusion, our data on spatio-temporal immunophenotyping of D-type cyclins have several implications for cell cycle control, development and function of the epididymis. Future studies involving segment- or cell- specific gene inactivation by in vivo strategies (Gao and Zhang, 2007; Glaser et al., 2005; Kuhn et al., 2007; Lewandoski, 2007; Prawitt et al., 2004; Ristevski, 2005; Xia et al., 2006) should provide accurate knowledge on how various growth factor signaling pathways are coupled to regulate cell cycle control in the epididymis. Our studies provide expression evaluation of D-type cyclins as a potential biological endpoint for assessment of physical injury or exposure to various reproductive toxicants that often lead to changes in cell cycle events in the epididymis.

4. Experiment procedures

4.1. Mice

Postnatal mice at 7d, 14d, 21d, 42d were used for all the experiments. All mice were on a 129 SvEv/C57BL/6J genetic background. Mice were maintained on a 12 h: 12 h light/dark cycles and supplied with food and water *ad libitum*. Experiments with mice were performed per NIH guidelines and approved by the KU Medical Center Institutional Animal Care and Use Committee.

4.2. Western blot analyses

Mice were killed by isoflurane (Minrad Inc., Bethlehem, PA) anesthesia; the epididymides were collected and immediately frozen on dry ice until further use. Protein extracts were prepared by homogenization in tissue RIPA lysis buffer (Santa Cruz Biotechnology, Inc.) containing protease inhibitors (Sigma, St. Louis, MO) and phenyl methyl sulfonyl fluoride (Sigma, St. Louis, MO). Protein content was quantified by Coomassie blue dye (BioRad, Hercules, CA) method using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE). Approximately 20 µg of proteins were denatured in loading buffer containing SDS-

mercaptoethanol at 100 C for 5 minutes and separated on 12 % polyacrylamide gels using the BioRad MiniProtein gel apparatus at a constant voltage of 200V. The separated proteins along with standard protein markers were transferred onto PVDF membranes by previous methods, blocked in 5 % milk and incubated with appropriate rabbit polyclonal antibodies against cyclinD1 (SC-450), cyclin D2 (SC-593), cyclin D3 (SC-182), β -tubulin (SC-9104) as described (Ma et al., 2004). The protein-antibody complexes were visualized by a chemiluminescence method as described (Ma et al., 2004). All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For all immunoblots, β -tubulin immunoreactivity was used as a loading control. All antibodies were used at a dilution of 1:2,000. Western blot analysis was performed on three independent experiments.

4.3. Histology

Either whole epididymis (from mice at 7d, 14d, 21d, 42d) or individual regions including the caput, corpus or cauda (from mice at 42d) were dissected out and fixed in 10 % buffered formalin, pH 7.0 (Richard Allan, Kalamazoo, MI) over night at room temperature. The tissues were processed, paraffin-embedded, and 6 μ m sections were cut and stained with hematoxylin/eosin as described (Kumar et al., 1997; Ma et al., 2004). The slides were scanned using a Nikon microscope (Nikon Inc., Melville, NY) and the digital images were captured by Metamorph software (Molecular Devices, Sunnyvale, CA).

4.4. Immunohistochemistry

For immunohistochemical analyses, the tissues sections were de-paraffinized with xylene and processed through graded alcohol series and finally rinsed in PBS. The sections were incubated first in blocking buffer (Zymed's ready-to-use normal goat serum; Invitrogen, Carlsbad, CA) at room temperature, and then different primary antibodies were added. For negative controls, sections were incubated with normal serum, instead of primary antibodies. The antigen-antibody complexes were visualized by either a color reaction using Histochemistry kit (Invitrogen, Carlsbad, CA) or immunofluorescence using FITC or RITC conjugated appropriate secondary antibodies as described (Wang H et al., 2005). A rat monoclonal antibody against mouse Ki67 (Dako Cytomation, Carpinteria, CA) was a gift from Dr. Leslie Heckert. All primary and secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used at a dilution of 1:200. The slides were observed using an Olympus epifluorescence microscope (Olympus America Inc., Center Valley, CA) and the images were digitally captured using the Metamorph software. For each primary antibody, multiple sections from at least three mice were analyzed.

4.5. Statistical analysis

Statistical analysis was done by ANOVA followed by Turkey's post-hoc test using Prism 4 program (OS X Mac version). A *P* value < 0.05 was considered significant.

Highlights

- Expression of D-type cyclins in the postnatal mouse epididymis was analyzed.
- D-type cyclins show segment and cell specific pattern with minimal overlap.

- Distinct pattern of expression of D1 and D3 before puberty suggests their role in cell proliferation.
- Expression of D-type cyclins in adult epididymis despite minimal cell proliferation reveals their possible roles independent of those in cell cycle.

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Abbreviations

FITC	Fluorescein isothiocyanate
RITC	Rhodamine isothiocyanate

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Fig. 1. Western blot analysis of D-type cyclins in the postnatal epididymis

A: Distinct profiles of expression of cyclins D1, D2 and D3 at different ages. **B:** Region-specific expression of cyclins D1, D2 and D3 in the epididymis at 42 days of age.

Expression of β-tubulin was used as an internal control. The molecular mass of each D-type cyclin is indicated. Two bands are seen in case of cyclin D1 and the major band is at 37KDa (arrows). The data in panels A and B from three independent sets of experiments were quantified by densitometry and shown in panels C and D respectively. Statistical analysis was done by ANOVA followed by Turkey's post-hoc test. The overall *P* values for panel C

data: D1=0.01; D2= 0.02 and D3 = 0.09. The overall P value for panel D data: D1= 0.02; D2 = 0.04 and D3 = 0.34. * $P < 0.05$ vs. 7d in panel C or vs. Caput in panel D; a and b denote $P < 0.05$ vs. 14d or 21d in panel C.

Fig. 2. Expression of cyclin D1

Pre-pubertal expression is represented (7d-21d). At 7d, expression of cyclin D1 was mainly detectable in epithelium of cauda (c) but only in few cells in epithelium of the caput and corpus epididymis (a and b). Higher levels of D1 expression were found in all three regions by 14d (d-f) and remained persistent up to 21d (g-i). Arrows indicate expression in principal cells. St: stromal cells. Bar represents 100 μ m. The inset in panel c shows no immunoreactivity in the absence of cyclin D1 antibody in a snap shot of a negative control section. Bar represents 100 μ m.

Fig. 3. Expression of cyclin D2

Pre-pubertal (7d-21d) expression of cyclin D2 is shown. Arrowheads in (c and f) indicate basal cell-specific expression. Cyclin D2 expression was weak in the cytoplasm of principal cells at d7 - d21 (arrows in d-i). St: stromal cells. A negative control section shows no immunoreactivity in the absence of cyclin D2 antibody (inset in panel c). Bar represents 100 μ m.

Fig. 4. Expression of cyclin D3

Pre-pubertal expression of Cyclin D3 is indicated (7d-21d). Unlike cyclin D1 and cyclin D2, the expression of cyclin D3 was predominantly confined to the nuclei of interstitium at 7d (a-e). At 14d and 21d, cyclin D3 was expressed in the nuclei of clear cells (arrows in f and i) and cytoplasm of principal cells (e and f, h and i). St: stromal cells; E: epithelial cells. A negative control section shows no immunoreactivity in the absence of cyclin D3 antibody (inset in panel c). Bar represents 100 μ m.

Fig. 5. Segmental expression of D-type cyclins in the adult epididymis
A: The ten segments of the epididymis are shown in a hematoxylin/eosin-stained adult (42d) epididymis section. **B:** Low power photomicrograph shows expression of cyclins D1, D2 and D3 in each of the ten segments in the epididymis of a mouse obtained at 42d of age. **C:** Schematic comparison of segment-specific expression patterns of cyclins D1, D2 and D3 in the adult epididymis. Each color represents the expression of a D-type cyclin as indicated. Bar represents 100 μ m.

Fig. 6. Cell-specific expression of D-type cyclins in different segments of the adult epididymis
Cyclin D1 was detectable mostly in caput and corpus (expression in segments 1, 6 and 7, i.e., S1, S6 and S7 is indicated; a, d, and g). It was noticeably localized to the principal (thin black arrow in a, d, g, j and m) but not apical (thick arrow in a) or basal cells (red arrow in a, d, g, j, and m) of the caput and corpus epididymis. High levels of cyclin D2 were observed in principal (thin black arrow in e, h, k and n) and basal cells (red arrow in b, e, h, k, and n), but weakly in the apical cells (thick arrow in b). Clear cells did not appear to express cyclin D2 (arrowhead in h). Cyclin D3 was expressed highly in principal (thin arrows in c, f,

i, l, and o) and apical cells (thick arrow in c), but weakly in basal cells (red arrow in c, f, i, l and o). Similar to cyclin D2, its staining was absent in clear cells (arrowhead in i, l, and o). In the caput, cyclin D3 was mainly localized to the nuclei (c), but in the corpus and cauda, it was mainly expressed in the cytoplasm (f, i, l and o). Bar represents 100 μ m.

Fig. 7. Cellular localization of D-type cyclins in the cauda and corpus epididymis in the adult Immunofluorescence analysis indicates that although cyclins D1 and D3 are coexpressed in segments, S6 (corpus) and S8 (cauda) (a–f), cyclin D1 is mainly restricted to the nucleus (a and c; arrowhead in d and f). In contrast, cyclin D3 is localized to the cytoplasm (b and c; arrowhead in e and f). cyclin D2 is detected both in cytoplasm and nucleus in these segments (g and i; arrowhead in j and l). Note that within these two segments, clear cells do not express any of the three cyclins, D1 or D2 or D3 (arrows in d–f and j–l). Bar represents 100 μ m.

Fig. 8. Expression of Ki-67

Ki67 is markedly expressed in both epithelium and stromal cells of all three epididymal regions at 7d. Then expression of Ki67 gradually decreases by 21d. Ki67 immuno-positive cells are rarely seen in the epididymis by 42d. Arrow show epithelium cell. St, stromal cells. Bar represents 100 μ m.