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ORIGINAL ARTICLE

Male Health

Comparison of gene expression of the oncogenic Wnt/β-catenin signaling pathway components in the mouse and human epididymis

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β-catenin is an integral part of the Wnt signaling pathway and has been linked to tumorigenesis and multiple developmental processes. The high β-catenin expression with low tumor incidence in the human epididymis is thus intriguing. In the present study, the β-catenin gene and protein was found to be highly expressed in the murine caput epididymidis, and the protein mainly localized along the lateral plasma membranes of adjacent epithelial cells throughout both human and mouse epididymides. Furthermore, the adult mouse epididymis was found to express almost all the Wnt/β-catenin signaling pathway genes that were determined previously by our group in the human organ. Despite the differences in epididymal structure, the similar location of β-catenin and the high concordance of this pathway's components' gene expression in both the adult human and mouse epididymides make the mouse a suitable animal model for studying the anti-tumor mechanism of the epididymis. In addition, both the mRNA and protein expression of β-catenin shared a similar spatial expression as the mRNA of Ros1, a proto-oncogene and a key developmental regulator of the initial segment of the mouse epididymis. The observations on the parallel temporal expression of β-catenin and Ros1 during postnatal development raise the possibility that the canonical Wnt signaling pathway has an additional role in the postnatal development of mouse epididymis.

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Keywords: epididymis; postnatal development; tumorigenesis; Wnt/β-catenin pathway; β-catenin

INTRODUCTION

The Wnt signaling pathway is one of the several evolutionarily conserved signal transduction pathways. Wnt signals transduce at least two distinct pathways: a well-established canonical or Wnt/β-catenin pathway, and a noncanonical β-catenin-independent pathway.[1] The canonical Wnt/β-catenin signaling pathway is triggered by the binding of a typical Wnt to a cognate receptor of the Frizzled (FZD) family, and ultimately results in the stabilization of β-catenin, which then enters the nucleus to form a transcriptional complex with T-cell factor (TCF) or lymphoid enhancer factor (LEF) to activate the expression of target genes such as *c*-Myc and cyclin D1.[2] The Wnt/β-catenin signaling pathway plays an essential role in tumorigenesis.[3] Almost 50% of all human cancers are associated with hyperactivation of the canonical Wnt pathway or dysregulation of specific components of this pathway.[4]

The epididymis, which develops from the Wolffian duct, is an important organ of the male reproductive system that functions in sperm maturation, transport, concentration, protection, and storage.[5] The human epididymis is known to be a cancer-resistant organ,[6] however, it expresses a complete set of Wnt/β-catenin pathway genes, notably with much higher β-catenin expression than the colorectal carcinoma cell line HCT116 that already possesses elevated β-catenin.[1] Such unexpected results make the human epididymis a relevant model for revealing the inhibitory mechanisms of a normal organ against tumorigenesis,[1,6] which may throw light on our understanding and clinical treatment of tumors or cancers. Other than the "fertile human" caput epididymidal cell line 1 (FHCE1),[7] no animal model has been shown to be suitable for such research.

Wnt/β-catenin signaling can also regulate the development, renewal, and regeneration of many organs.[8] It has been shown that β-catenin increases with age throughout the entire rat epididymis and its expression correlates well with the formation of the blood-epididymis barrier.[9] Wu *et al*. have demonstrated that β-catenin mRNA is negatively regulated by miR-200a during postnatal rat epididymal development.[10] Evidence for the role of β-catenin in postnatal mouse epididymal development is limited since Wolffian duct-specific deletion of β-catenin causes early postnatal lethality in mice, which possess an abnormal, dilated epididymis.[8] In summary, the role of β-catenin during epididymal development is still obscure, and it is therefore of interest to study the role of β-catenin during postnatal epididymal development.

The proto-oncogene Ros1 (also known as c-*ros*) is another gene involved in both tumorigenesis and male reproductive tract

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development,[11] and it has been identified in human, mouse, and rat epididymides.[12–14] Previous studies have shown that in contrast to the usual postnatal down-regulation in other normal tissues, Ros1 is upregulated in the proximal caput epididymidis around puberty.[11,13] Moreover, Ros1 deletion leads to the failure of development of the initial segment (IS), coupled with male infertility.[13] The involvement of Ros1 as well as β-catenin in both oncogenesis and development led us to wonder whether there is any functional connection between these two molecules during postnatal mouse epididymal development.

In the present study, the expression of Wnt/β-catenin signaling component genes in the mouse epididymis was examined by RT-PCR, and compared with that of the human. The expression of β-catenin protein in mouse and human epididymides was quantified by Western blots and localized by immuno-histochemical staining. We discuss how suitable the mouse is as an animal model for investigation of anti-tumor mechanisms in the epididymis *in vivo*.

MATERIALS AND METHODS

Animals and tissues

All animal and human experimental procedures were approved by the Ethics Committee of Shandong Research Centre of Stem Cell Engineering (Yantai, Shandong, China). Animals were kept under standard conditions of temperature (22°C) and light (12L/12D) and had access to water and food *ad libitum*. Organs were obtained from developing (7-, 14-, 16-, 21-, 32-, 49-, 70-day-old) and adult (90-day-old) wild-type Kunming mice (LuYe Pharmaceutical Company, Yantai, Shandong, China). Human epididymides from three organ donors (27–32 years old), who had died in car accidents and who did not have any history of pathology that could affect reproductive functions, were collected while artificial circulation maintained organs assigned for transplantation. Each epididymis was dissected into caput, corpus, and cauda regions according to Li *et al*.[15] The mouse caput contained the initial segment (IS) but excluded the efferent ducts.[16]

Western blotting and quantification

For Western blots, murine epididymal proteins were pooled from different developmental ages (six animals per age group) and the other organ proteins were pooled from five adult mice. Total proteins were extracted, and Western blot analysis was performed as described previously.[17] Immuno-blots were developed with ECL reagent (Pierce Biosciences, Rockford, IL, USA) according to the manufacturer's instructions. There were no nonspecific bands on the gels when incubated with antibodies against β-catenin and Gapdh (data not shown).

For quantification, densitometric analysis was performed on the gray level intensity of target bands derived from scanned films, processed by using Gene Tools image analysis software (GeneTools, version 4.02; Syngene, Cambridge, UK) according to Wang *et al*.[17] The primary antibodies used were β-catenin antibody (9587, Cell Signalling Technology, Danvers, MA, USA) and Gapdh antibody (2118, Cell Signalling Technology).

RNA isolation and RT–PCR

Epididymides from six mice at each developmental age were pooled for RNA isolation. Total RNA was extracted with RNAiso Plus reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. The concentration of RNA was determined by spectrophotometry (Nanodrop 2000/2000C, Thermo Scientific, Wilmington, USA). Two microgram total RNA was reverse-transcribed with ReverTra Ace (TOYOBO, Osaka, Japan). Taq polymerase (TaKaRa) was used for PCR and the reaction conditions were: a 5 min initial denaturing at 94°C, followed by an appropriate number of cycles of denaturing at 94°C for 30 s, annealing at different temperatures for 30 s (**Supplementary Table 1**) and extension at 72°C for 30 s, and the final extension step at 72°C for 10 min. The forward and the reverse primers were chosen to span several introns to avoid genomic DNA amplification (**Supplementary Table 1**). The PCR products were separated on a 1% (w/v) agarose gel, and visualized by ethidium bromide staining, with a 2 kb DNA ladder (TaKaRa) to indicate the size of the products. Amplification of the housekeeping gene *Gapdh* was used as positive control. A negative control for amplimer contamination was made from a complete PCR reaction mixture without cDNA.

Quantitative PCR

Quantitative PCR analysis was run in a Roter-Gene Q (QIAGEN, Hilden, Germany) with the Platinum SYBR Green qPCR SuperMix-UDG kit (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. Cycling conditions were: 50°C for 2 min; 95°C for 5 min; followed by 40 cycles of 95°C, 10 s and 60°C, 45 s. Melting curve analysis and agarose gel electrophoresis were used to confirm the specific PCR products. *Gapdh* was used as an endogenous reference. The $2^{-\Delta\Delta Ct}$ method was used to calculate the differences of the expression level of, *Ctnnb1* and *Ros1* during the postnatal development period. All experiments pooled from six mice were run in triplicate to indicate intra-assay variation. The primer sequences were as follows: *Ctnnb1* forward (F): 5'-CTGCTCATCCCACTAATGTC-3', reverse (R): 5'-CTTTATTAACTACCACCTGGTCCT-3'; *Ros1* (F): 5'-CTGTGGATTCAGTTGGTGGCTATC-3', (R): 5'-ATTGTCCTGCACCAGCCAATAC-3'; *Gapdh* (F): 5'-TGTGTCCGTCGTGGATCTGA-3'; (R): 5'-TTGCTGTTGAAGTCGCAGGAG-3'.

Immuno-histochemistry and quantification

Immuno-histochemistry was performed according to the method of Zhu *et al*.[18] Dilutions of primary and secondary antibodies were anti-β-catenin 1:50 and horseradish peroxidase-conjugated goat anti-rabbit IgG 1:200. The staining specificity was demonstrated by incubation of the section with rabbit IgG instead of primary antibody. Sections were examined with bright-field microscopy (DM LB2, Leica, Nussloch, Germany).

RESULTS

Protein expression of β-catenin in different tissues of the mouse

The protein expression of β-catenin in mouse tissues was assessed by Western blot analysis (**Figure 1a**). Quantification showed that β-catenin was easily detected in the caput, corpus, and cauda epididymidis, testis, heart, lung, and brain; whereas in the liver, spleen, and kidney, β-catenin protein levels were very low (**Figure 1b**). Notably, the caput appeared to express more β-catenin than other samples (**Figure 1b**).

Gene expression of the Wnt/β-catenin signaling components in the adult mouse epididymis

Wnt/β-catenin signaling pathway component expression was assessed by RT-PCR (**Figure 2**). There were (i) six canonical *Wnts* (*Wnt2*, *Wnt2b*, *Wnt3*, *Wnt3a*, *Wnt9b*, and *Wnt10a*), (ii) nine *Wnt* receptor genes (*Fzd1*, *2*, *3*, *4*, *6*, *7*, *8*, *9*, and *10*), (iii) almost all major components essential for downstream signal transduction (*Dvl*, *Axin*, *Apc*, *Ck1α1*, and *Gsk3α/β*), (iv) three *Dickkopf* (*DKK*) family members (*Dkk1*, *Dkk3*, and *Dkk4*) and (v) the transcription factors (*Lef1* and *Tcf4*) in the mouse epididymis.

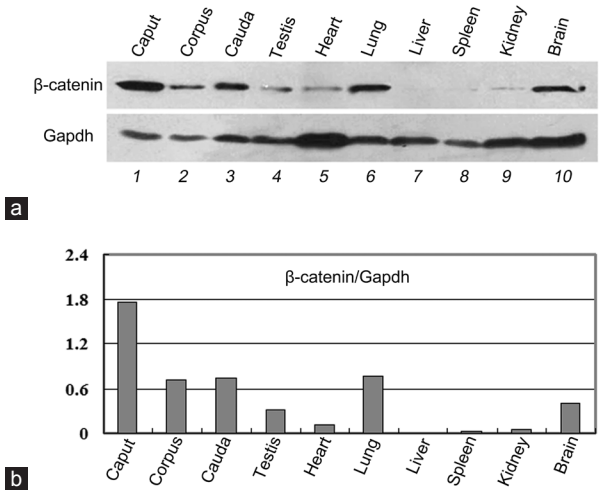

Figure 1: Protein expression of β-catenin in mouse organs. Protein expression (**a**) and the quantification (**b**) of β-catenin in various mouse tissues by Western blots and densitometry. Each value is the mean absorbance of triplicates of protein pools from each organ.

Localization and quantification of β-catenin in adult mouse and human epididymides

Immuno-staining showed that β-catenin was highly expressed throughout mouse and human epididymides, with the highest signal along the lateral plasma membranes (*arrows* **Figure 3a**), less in the cytoplasm and little in the nuclei of the adjacent epithelial principal cells in the caput, corpus, and cauda regions of both species (**Figure 3a**). No hyperplasia of the epididymal epithelium was found in either human or mouse (**Figure 3a**).

Quantification of β-catenin expression in different regions of both human and mouse epididymis showed a declining expression pattern from caput to cauda (**Figure 3b**), which was consistent with the immuno-histochemistry results (**Figure 3a**). Western data of human epididymal β-catenin were quantified from our previous results.[1]

Changes in β-catenin expression during postnatal development in the mouse

Postnatal immuno-cytochemical studies revealed that β-catenin was expressed by postnatal day 7 and was mainly localized along the lateral plasma membrane between the adjacent epithelial cells throughout the whole epididymis (**Figure 4**). Whereas positive staining was just discernible from days 7 to 14, it obviously increased from days 16 to 32 in all regions.

qPCR results revealed that β-catenin and Ros1 shared a similar expression pattern in the caput epididymidis: low on day 7, increasing gradually to reach a peak on day 32, and then beginning to decrease until day 90 (**Figure 5a**). Western blot results of β-catenin in the caput (**Figure 5b**) were consistent with those from qPCR (**Figure 5c**).

DISCUSSION

In the present study, β-catenin was found to be highly expressed in both human and mouse epididymides, especially in the caput. In addition, six canonical Wnts (Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt9b, and Wnt10a), nine Wnt receptor genes (Fzd1, 2, 3, 4, 6, 7, 8, 9, and 10), almost all major components essential for downstream signal transduction (Dvl, Axin, Apc, Ck1α1, and Gsk3α/β), three Dickkopf (DKK) family members (Dkk1, Dkk3, and Dkk4) and the down-stream transcription factors (Lef1 and Tcf4) of the canonical Wnt pathway[19,20] were identified in the mouse epididymis. Despite such high β-catenin expression

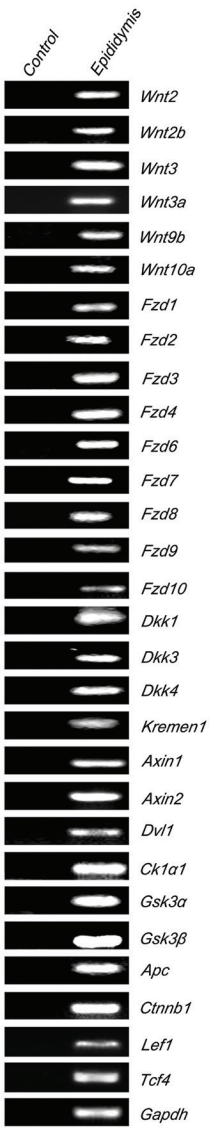

Figure 2: Gene expression of the Wnt/β-catenin signaling genes in the adult mouse epididymis. Wnt/β-catenin signaling components in the entire mouse epididymis were analyzed by RT-PCR, with *Gapdh* as an internal control. Results of one representative experiment of triplicates. The negative control was made from a complete PCR reaction mixture without cDNA.

and all the components of the Wnt-signaling pathway, there was no hyperplasia in the epididymal epithelium of either human or mouse, suggesting inhibition of the pathway. Furthermore, β-catenin was mainly immuno-localized along the lateral plasma membranes of adjacent epithelial cells, not in the nuclei, throughout the epididymis of both species. This raises the possibility that membrane accumulation of β-catenin is a key epididymal anti-tumor property since after release from membrane, accumulated β-catenin can translocate to the nucleus and cause cancer in cancer-prone organs.[3] Cell adhesion molecules such as E-cadherin and CEACAM1 may play essential roles in anti-tumorigenesis of the epididymis, because they have been shown to be tumor suppressors in various epithelial tumors, and they can directly interact with β-catenin, anchoring it to the cell membrane.[21,22]

We suggest that such anchoring the highly expressed β-catenin on the membrane forms the first barrier for the epididymis against tumorigenesis. In the rat epididymis, when released from E-cadherin

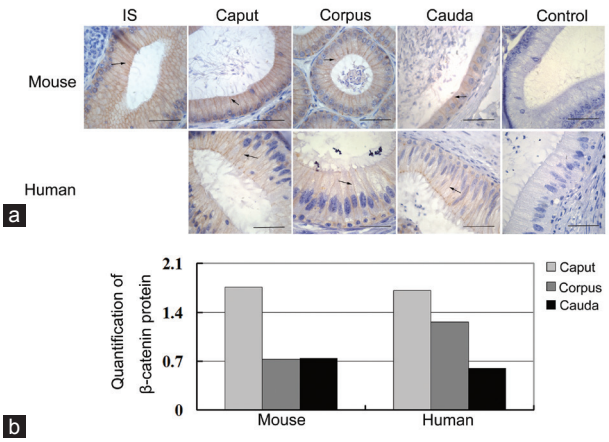

Figure 3: Localization and quantification of β-catenin in mouse and human epididymides. (**a**). Representative immuno-localization of β-catenin protein in different regions of three adult mouse and three human epididymides. Negative controls with rabbit IgG instead of primary antibody were done in all regions but shown only for the caput region. Scale bar, 40 μm. IS, initial segment. (**b**) Quantification of Western results of β-catenin in different regions of the mouse and human epididymides. Each value is the mean absorbance of triplicates of protein pools from each region. Human data from Wang *et al*.[1]

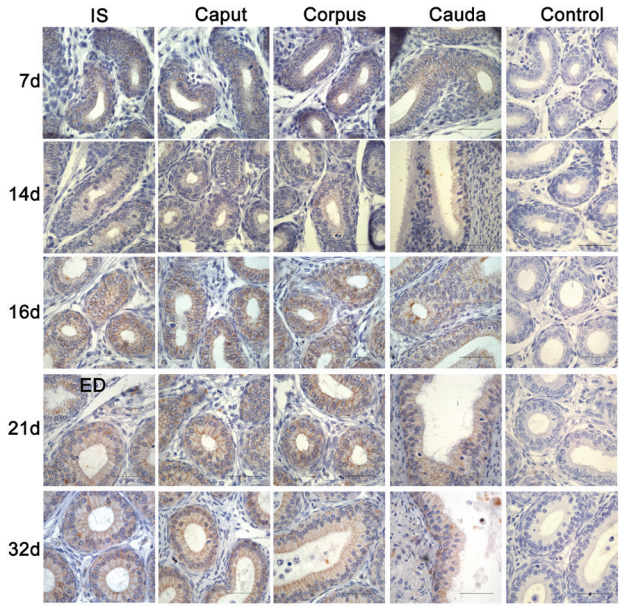

Figure 4: Localization of β-catenin in different mouse epididymal regions during postnatal development. The ages (expressed in days (d)) are shown on the left, and the regions of the epididymis are shown at the top. Scale bars, 40 μm. Negative controls with rabbit IgG instead of primary antibody were done in all regions but only shown for the caput region. Representative epididymal sections from one of the three mice. IS: initial segment; ED: efferent ducts.

after castration, β-catenin accumulates only in the cytoplasm of the epithelium, not the nucleus,[9] which suggests that there may be a second anti-tumor barrier in the cytoplasm which prevents unbound β-catenin from entering the nucleus and causing subsequent cell transformation.

Use of the Catnb$^{+/\Delta ex3}$ mouse model has confirmed that the epididymis in this species is also cancer-resistant.[23] Such transgenic mouse can express excessive stabilized β-catenin, which still has the ability to bind to the cell adhesion molecules E-cadherin and CEACAM1[22,24] and not be degraded. Whereas this model exhibits high-grade intra-epithelial neoplasia in the prostate,[23] it only causes hyperplasia and squamous metaplasia in the epididymis, strongly supporting the hypothesis of a second anti-tumor barrier in the epididymis. Collectively, there could be a comprehensive mechanism which suppresses the oncogenic activity of β-catenin and keeps the epididymis a tumor-resistant organ.

Through comparing β-catenin protein expression and the Wnt/β-catenin pathway gene components of mouse and human epididymides, we found that β-catenin was highly expressed in both the human and mouse caput, and the localization of mouse β-catenin in the epididymis was similar to that in the human. Despite the same sub-cellular localization of β-catenin in the rat epididymal epithelium as in the mouse, there is more β-catenin in the corpus and cauda than the caput.[9] In the mouse, gene expression of the canonical Wnt/β-catenin pathway components in the epididymis resembles that of the human, except for some differences in the expression of *DKKL1*, *Fzd2*, *8*, *9*, *10*, and *Wnt10a* (**Table 1**). *DKKL1* is only detected in the human, but it is not a modulator of the canonical Wnt/β-catenin signaling pathway,[25] and the mouse epididymis expresses a greater variety of Fzds than the human, such as *Fzd2*, *8*, *9*, and *10*.

Wnt may signal through different Fzds, and the specific Wnt-Fzd interaction may dictate which signaling pathway is activated.[26] Fzd2, 8, and 10 can help trigger the canonical WNT/β-catenin signaling pathway through Wnt3a, and Fzd9 through Wnt2.[26] In the present study, human and mouse shared almost the same canonical Wnts expression, except *Wnt10a*, so both human and mouse epididymides may share the same mechanism against Wnt/β-catenin signaling activation-induced tumorigenesis. For these reasons, the mouse may be a good animal model for the study of oncogenic suppression in the human epididymis.

For the mouse, our results suggested that the expression of β-catenin, both protein and mRNA, increased during postnatal epididymal development. However, conflicting reports about the expression of β-catenin during rat postnatal epididymal development have been published. One study has demonstrated an increased expression of β-catenin protein,[9] whereas Wu *et al*.[10] has shown a decreased expression of β-catenin mRNA. Our results showed that expression of both β-catenin protein and mRNA increased during puberty in the mouse, which may be a response to the parallel increase of androgen.[9] However, the levels of β-catenin did not remain constant with androgens thereafter; there was a gradual decrease, which may be associated with the cessation of epididymal differentiation.[27]

The increased expression of β-catenin between 16 and 32 days precisely spanned the period when the IS begins to become recognizable in its characteristic adult form. β-catenin regulates the postnatal development of many organs,[28] and Wolffian duct-specific deletion of β-catenin in mouse leads to an abnormal, dilated epididymis and vas deferens,[8] so β-catenin could be involved in postnatal IS differentiation of the mouse epididymis. The temporal expression of Ros1 mRNA (a key developmental regulator of the IS)[13] was found here to parallel closely that of β-catenin mRNA. Previous studies on cell cultures have shown that Ros1 down-regulates Wnt/β-catenin in B-cell lines in culture, since β-catenin expression is upregulated after silencing of Ros1 by Ros1 siRNA,[29] however, our results do not indicate such a relationship between β-catenin and Ros1 in the epididymis.

However, since their expression was in parallel during development, these proteins may play co-ordinated roles. Relationships between β-catenin and Ros1 in IS differentiation need

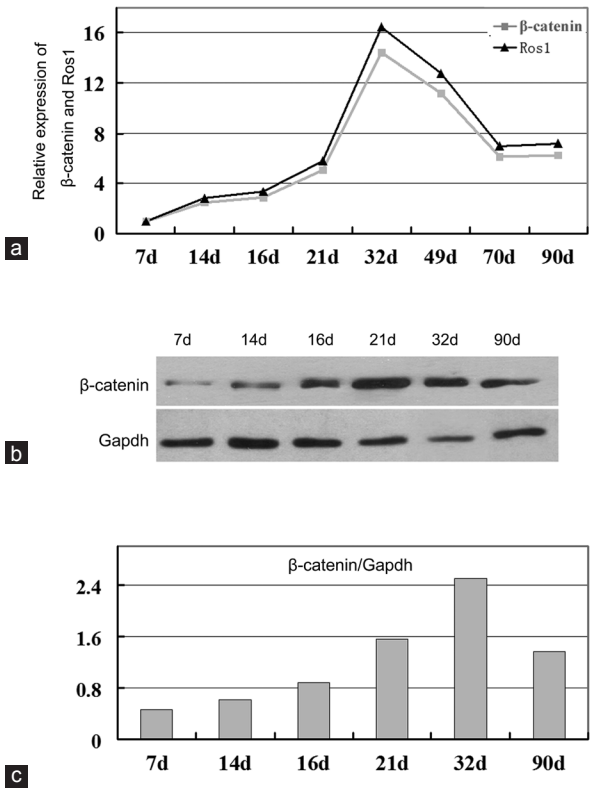

Figure 5: Expression of β-catenin mRNA and protein in the mouse caput epididymidis (including IS) during postnatal development. (**a**) Quantification of β-catenin and Ros1 mRNA expression in the caput epididymidis from postnatal days 7 to 90. The average level of β-catenin and Ros1 mRNA expression on day 7 was set as 1.0. Results are expressed as mean of triplicates of RNA pools at each time. (**b**) Western blot analysis of β-catenin expression from days 7 to 90 in the caput region. Results of one representative experiment of triplicates. (**c**) The relative amount of β-catenin protein in the mouse caput epididymidis at different stages of development. Each value is the mean absorbance of triplicates of protein pools at each time.

further study. β-catenin may be involved in IS differentiation through association with the androgen receptor (AR), which is also required for the formation of the IS.[30] It has been shown that β-catenin is a co-regulatory protein of AR, and it potentiates AR signaling in an androgen-dependent fashion in prostatic cells.[31] The cross-talk between the AR and β-catenin in postnatal epididymal development remains to be studied.

CONCLUSION

Our results suggest that the oncogenic Wnt/β-catenin pathway may be inhibited and that components of it may play a role in epididymal development. Despite the differences in epididymal structure, the similar location of β-catenin and the high concordance of Wnt/β-catenin pathway's components' gene expression in both human and mouse epididymides make the mouse a suitable animal model for studying anti-tumor mechanisms of the human epididymis. Its use may help to unmask the potential mechanisms whereby the epididymis evades cancer *in vivo*. The mechanism that anchors β-catenin to the membrane may form the first barrier for the epididymis against tumorigenesis. Further study into how cell adhesion molecules play roles in anchoring β-catenin in epididymis will help us to understand the regulation of Wnt/β-catenin signaling pathway.

Table 1: Gene expression of the Wnt signaling pathway in both the human and mouse epididymis

Gene symbol	*Accession numbers Human/mouse*	*Human epididymis*	*Mouse epididymis*
WNT1	NM_005430.3/NM_021279.4	–	–
WNT2	NM_003391.2/NM_023653.5	√	√
WNT2B	NM_004185.4/NM_009520.3	√	√
WNT3	NM_030753.4/NM_009521.2	√	√
WNT3A	NM_033131.3/NM_009522.2	√	√
WNT8A	NM_001300938.1/NM_009290.2	–	–
WNT8B	NM_003393.3/NM_011720.3	–	–
WNT9A	NM_003395.2/NM_139298.2	–	–
WNT9B	NM_003396.1/NM_011719.4	√	√
WNT10A	NM_025216.2/NM_009518.2	–	√
WNT10B	NM_003394.3/NM_011718.2	–	–
FZD1	NM_003505.1/NM_021457.3	–	√
FZD2	NM_001466.3/NM_020510.2	–	√
FZD3	NM_017412.3/NM_021458.2	√	√
FZD4	NM_012193.3/NM_008055.4	√	√
FZD5	NM_003468.3/NM_022721.3	–	–
FZD6	NM_003506.3/NM_008056.3	√	√
FZD7	NM_003507.1/NM_008057.3	√	√
FZD8	NM_031866.2/NM_008058.2	–	√
FZD9	NM_010246.1/NM_003508.2	–	√
FZD10	NM_007197.3/NM_175284.3	–	√
CTNNB1	NM_001904.3/NM_007614.3	√	√
DKK1	NM_012242.2/NM_010051.3	√	√
DKK2	NM_014421.2/NM_020265.4	–	–
DKK3	NM_001018057.1/NM_015814.2	√	√
DKK4	NM_014420.2/NM_145592.2	√	√
DKKL1	NM_014419.3/NM_015789.3	√	–
GSK3alpha	NM_019884.2/NM_001031667.1	√	√
GSK3beta	NM_002093.3/NM_019827.6	√	√
DVL1	NM_004421.2/NM_010091.4	√	√
CK1alpha 1	NM_001025105.2/NM_146087.2	√	√
Axin1	NM_003502.3/NM_009733.2	√	√
Axin2	NM_004655.3/NM_015732.4	√	√
APC	NM_007462.3/NM_001127511.2	√	√
KREMEN1	NM_032045.4/NM_032396.3	√	√
KREMEN2	NM_024507.3/NM_028416.2	–	–

–: absent; √: present

AUTHOR CONTRIBUTIONS

KW, NL, CHY, and TGC were responsible for the concept and framework of the paper. KW and NL wrote the manuscript which was revised by CHY and TGC. KW planned and performed the experiments. NL, JL, YL, and FJL carried out RT-PCR and Western blot. XXL, WTW, and HS carried out IHC. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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