Calcium- and integrin-binding protein-1 is down-regulated in the sperm of patients with oligoasthenozoospermia

CIB1 expression in patients with oligoasthenozoospermia

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Abstract

Purpose The aim of this study was to determine whether altered expression and distribution of calcium- and integrin-binding protein-1 (CIB1) is involved in the pathogenesis of patients with oligoasthenozoospermia.

Methods Sperm samples were obtained from 25 infertile Chinese men who had failed to achieve conception after a period of 1–2 y and had been referred to the Reproductive Laboratory of the second hospital affiliated to the Shandong University of Traditional Chinese Medicine. Participants were divided into two groups: oligoasthenozoospermia ($n=13$) and asthenozoospermia ($n=12$); as a third group, fertile men ($n=19$) were included as controls. The expression levels of mRNA and protein levels of CIB1 and cyclin-dependent kinase 1 (CDK1) were measured using qRT-PCR and western blotting.

Results mRNA and protein expression levels of CIB1 were decreased in the oligoasthenozoospermia patients. Interestingly mRNA and protein expression levels of CDK1 were increased in the oligoasthenozoospermia patients.

Conclusion The results of the present study indicate that that CIB1 may be involved in the pathogenesis of oligoasthenozoospermia by the CDK1 signaling pathway.

Keywords CDK1 · CIB1 · Male infertility · Oligoasthenozoospermia · Sperm

Introduction

Infertility is a worldwide reproductive health problem that affects about 15 % of couples [1], and the marked decrease in fertility rates in many industrialized countries demand further scientific attention [2]. Half of infertility cases are due to male factors, and 75 % of such cases are idiopathic [3]. Male infertility is a complex problem, involving genetic and epigenetic factors [4]. In most case, male infertility is accompanied by idiopathic oligoasthenozoospermia (subjects with a sperm concentration $<15 \times 10^6/\text{ml}$ and sperm progressive motility $<32\%$) and asthenozoospermia (subjects with a sperm concentration $\geq 15 \times 10^6/\text{ml}$ and sperm progressive motility $<32\%$), according to the classification system of the World Health Organization (WHO) Manual from 2010 [5].

Currently, idiopathic oligoasthenozoospermia patients are treated with gonadotrophin, which induces an increase in the pregnancy rate or a decrease in sperm fragmentation [6, 7], but the factors and mechanisms underlying oligoasthenozoospermia remain unclear.

Ca^{2+} signaling is of particular significance in sperm cells, where it is a central regulator in many key activities including capacitation, hyperactivation, chemotaxis and the acrosome reaction [8, 9]. Impairment of Ca^{2+} signaling in sperm is associated with male subfertility [10]. Cells respond to changes in Ca^{2+} levels mainly by activation of EF-hand proteins, including those belonging to the CIB family. The human CIB protein family is comprised of four homologs (CIB1, -2, -3, and -4), CIB1 may be involved in integrin αIIb activation, the DNA damage response, apoptosis, embryogenesis, and regulation of Ca^{2+} signals [11, 12]. Recently, the in vivo

Capsule CIB1 mRNA and protein expression patterns oppose those of CDK1 in sperm of oligoasthenozoospermia patients. CIB1 may be involved in the pathogenesis of oligoasthenozoospermia by the CDK1 signaling pathway.

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role of CIB1 was elucidated by generation of transgenic mice. *Cib1*-null males were sterile due to disruption of the haploid phase of spermatogenesis. *CDK1* was found to be overexpressed in the testes of *Cib1*^{−/−} male mice and cells isolated from testis tubules, and it is confirmed that its overexpression was unique to the testis not a general phenomenon, it was considered that CIB1 may interact with CDK1, albeit transiently [13]. In our previous study, *CIB1* was widely expressed in different tissues in sheep, with the highest level being observed in the testis [14]; moreover, another member of this family, CIB4, may be linked to the high fecundity of a sheep breed indigenous to China [15]. These studies indicated that CIB1 may play an important role in human male fertility.

This study investigated the expression of *CIB1* in oligoasthenozoospermia patients and aimed to determine whether CIB1 interacts with cell cycle-regulatory proteins, in order to further elucidate the etiopathogenetic pathway of oligoasthenozoospermia.

Materials and methods

Patients

This study was approved by the ethics review board of the Second Hospital affiliated to the Shandong University of Traditional Chinese Medicine, China. The study population consisted of 22 infertile Chinese men who had failed to achieve conception after a period of 1–2 y and who had been referred to the Reproductive Laboratory of the Second Hospital affiliated to the Shandong University of Traditional Chinese Medicine. A questionnaire was used to collect information from subjects, including information regarding their age, height, weight, personal background, lifestyle factors, occupational and environmental exposures, genetic risk factors, sexual and reproduction status, medical history, and physical activity. Men who had ejaculatory dysfunction, a medical history of risk factors for infertility (e.g., postvasectomy, orchidopexy, or varicocele), immune-related infertility, and who had received treatment for infertility (e.g., hormonal treatments), or who had documented infection, genetic disease, or occupational exposure to agents suspected to be associated with male reproduction were excluded from the study. The controls included 22 fertile male volunteers from the early pregnancy registry of the same hospital, whose partners were in the second trimester of pregnancy.

The infertile men were divided into two subgroups according to sperm concentration and motility, based on WHO reference values (2010) [5], viz. oligoasthenozoospermia ($n=11$), and asthenozoospermia ($n=11$). The diagnosis of oligoasthenozoospermia and asthenozoospermia was confirmed by at least two consecutive spermograms performed on ejaculated semen collected after 3–5 days of sexual abstinence.

Sperm collection, purification, and storage

Semen samples were obtained after a recommended 3–5 days of sexual abstinence and were allowed to liquefy for 30–60 min at 37 °C. Semen analysis was performed using the computer-assisted semen analysis system (CASA, WLJY-9000; Weili New Century Science and Tech Dev, Beijing, China) according to the criteria of the WHO (2010) for semen volume, sperm concentration, and sperm motility.

Sperm samples were processed by discontinuous gradient centrifugation through a sperm separation medium upper layer (Irvine Scientific, Santa Ana, CA, USA) to separate mature sperm from somatic cells and immature diploid spermatocytes [16]. A semen sample (1–2 mL) was gently layered over the top of triple-volume, prewarmed (37 °C) sperm separation medium upper layer and centrifuged at 200×*g* for 30 min at room temperature. The sperm pellet was transferred into 15-mL centrifuge tubes and washed twice with sperm washing medium (Quinn's, Trumbull, CT, USA). Successful separation from round cells was assessed by light microscopy. Purified sperm was mixed with 1 mL TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) while shaking the tube for 15 s, and samples were then stored at −80 °C until needed.

RNA extraction and cDNA synthesis

Total RNA was extracted from spermatozoa using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's protocol. The purified RNA samples were resuspended in 60 µL of RNase/DNase-free water. RNA was quantified spectrophotometrically at 260 nm and the quality of RNA was checked using a 1 % 3-(N-morpholino) propane-sulfonic acid (MOPS)-formaldehyde gel. An M-MLV Reverse Transcriptase kit (Promega, Madison, WI, USA) was used to synthesize cDNA using 0.5 µL of the purified RNA and oligo dT₍₁₈₎ primers (Invitrogen, Carlsbad, CA, USA). The transcribed cDNA was assessed by PCR for the β-actin-encoding gene (*ACTB*; GenBank accession no. NM 001101), performed using paired primers. The conditions for PCR were: denaturation at 94 °C for 3 min (first cycle); then, 38 cycles each consisting of 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 15 s, followed by 72 °C for 5 min in the last cycle. The amplified product was inspected by gel electrophoresis.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed to determine the expression levels of *CIB1* and *CDK1* in human sperm samples. Total RNA was isolated from the sperm of three groups of patients, with four patients in each group. RNA extraction and reverse transcription were performed as described above. *CIB1*,

CDK1 and *ACTB* were amplified using the following specific primers: *CIB1* (sense: 5'-GAACCATCAACCTCTCT-3', antisense: 5'-GGGTCAAACCTTCTAAAC-3'), *CDK1* (sense: 5'-GGGCACTCCCAATAATG-3', antisense: 5'-CGAGAGCA AATCCAAGC-3'), and *ACTB* (sense: 5'-ATCATGTTTGAG ACCTTCAACA-3', antisense: 5'-CATCTCTTGCTCGAAG TCCA-3'). All gene-specific primers were designed to span at least one intron. We had previously determined the optimal conditions for RT-PCR [17]. The RT-PCR cycle numbers for individual genes were first tested to ensure that the PCR cycle number was in a linear amplification range. The PCR amplifications were carried out for 36, 35, and 31 cycles for *CIB1*, *CDK1*, and *ACTB*, and the annealing temperatures were 41 °C, 56 °C, and 51 °C, respectively. *ACTB* expression showed no significant difference among different sperm samples; hence, it was used as an endogenous control gene.

PCR products were separated on a 1.5 % agarose gel containing ethidium bromide and visualized under ultraviolet light, and photographed using a ChemiDOC (Bio-Rad, Hercules, CA, USA). Band intensity was analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed on an ABI PRISM 7700 Real-Time PCR System (Applied Biosystems, ABI, USA) with 10×SYBR Green I (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's protocol. In brief, the 25-μL PCR mixture included 1 μL cDNA, 12.5 μL 2×mix, 1 μL 10×SYBR Green I, 9.5 μL ddH₂O, and 10 μM forward and reverse primers. Cycling conditions included 2 min at 94 °C; then, 35 cycles each consisting of 30 s at 94 °C, 30 s at the appropriate annealing temperature, and 30 s at 72 °C. We used *ACTB* analysis in parallel for each run as an internal control. Standard curve arbitrary units were set at 1 for the undiluted PCR amplified products and dilutions of 1, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰, 10⁻¹¹, 10⁻¹², 10⁻¹³ and 10⁻¹⁴ were made. Standard curves were generated by plotting the threshold cycle numbers (*C_T*) vs. the log₁₀^{copies} of serially diluted PCR products. The slope, intercept, and correlation coefficient (*R*²) were determined by linear regression analysis using Microcal OriginPro Version 7.5 (OriginLab, Northampton, MA, USA). Each sample was run in triplicate to ensure quantitative accuracy, and the *C_T* values were averaged.

Western blot analysis

All sperm cells were lysed in RIPA lysis buffer (Dingguo, Beijing, China). Protein concentration was determined by the Lowry method with the absorbance read on a Q5000 spectrophotometer (Quwell, San Jose, CA, USA). The denatured samples were subjected to western blot analysis. A 25-μg aliquot

of the protein samples were separated by 12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 1.5 h at 120 V and the proteins were then transferred to nitrocellulose membranes (Pall, Ann Arbor, MI, USA). After blocking in 5 % fat-free milk, the membrane was incubated overnight at 4 °C with the primary anti-*CIB1* antibody (Abcam, Cambridge, MA, USA), *CDK1* (Abcam, Cambridge, MA, USA), followed by incubation for 2 h at room temperature with the secondary goat anti-rabbit IgG, which had been diluted in phosphate-buffered saline containing 5 % fat-free milk and 0.1 % Tween-20. The stained membranes were then visualized by an enhanced chemiluminescence reaction using ECL Plus (GE Healthcare, Fairfield, CT, USA). The western blotting experiments were repeated at least twice times for every sample with similar results.

Statistical analysis

All values are expressed as Mean±S.E.M. Differences between three groups were determined by one-way analysis of variance (ANOVA). Correlations between *CIB1* expression and clinical characteristics and *CDK1* expression in the study populations were evaluated using linear regression analysis. Fisher's exact test was used to test the differences in categorical variables such as smoking status. *P*-values<0.05 were considered statistically significant.

Results

Clinical characteristics of the study populations

The study contained 13 oligoasthenozoospermia patients, 12 asthenozoospermia patients, and 19 proven-fertile controls. There were no significant differences in age, smoking status, body mass index (BMI), sexual abstinence time, or total semen volume among the three groups. However, there were significant differences in sperm density, normal sperm morphology, and sperm viability among the three groups (*P*<0.01). Sperm density of the oligoasthenozoospermia group was lower than that in the asthenozoospermia and control groups; sperm viability in the oligoasthenozoospermia and asthenozoospermia groups were lower than that in the control groups, and normal sperm morphology of the oligoasthenozoospermia and control groups were higher than that in the asthenozoospermia group (Table 1).

CIB1 expression patterns oppose those of *CDK1* in the sperm of oligoasthenozoospermia patients

RT-PCR and qRT-PCR analysis demonstrated that the *CIB1* mRNA expression level in sperm from the control group was higher than that in the sperm of oligoasthenozoospermia patients (*P*<0.01; Fig. 1a–c), and the *CIB1* mRNA expression

Table 1 Baseline clinical and seminal features of the infertile patients and fertile controls

Variable	Oligoastheno-zoospermia (n=11)	Asthenozoospermia (n=11)	Controls (n=22)	P ^a	P ^b	P ^c
Age (years)	34.36 (3.64)	33 (5.23)	32.45 (4.44)	0.479	0.254	0.743
Smoking status, n				1.000 ^d	0.258 ^d	0.109 ^d
Ever	2	1	9			
Never	9	10	13			
BMI, kg/m ²	25.65 (4.4)	24.03 (2.17)	24.51 (3.28)	0.267	0.369	0.698
Sexual abstinence time, days	4.86 (1.21)	54.91 (1.32)	5.82 (2.44)	0.957	0.149	0.216
Semen total volume, mL	3.51 (1.10)	3.50 (1.10)	3.12 (0.78)	0.973	0.270	0.286
Sperm density, ×10 ⁶ /mL	12.20 (5.10)	36.79 (10.67)	70.07 (35.68)	0.033	0.000	0.001
Normal sperm morphology, %	15.61 (0.89)	14.41 (1.66)	16.41 (0.93)	0.018	0.067	0.000
Sperm viability, %	42.84 (17.56)	44.77 (13.51)	74.93 (16.68)	0.781	0.000	0.000
progressive motility, %	23.90 (10.37)	25.82 (7.86)	62.50(14.19)	0.710	0.000	0.000

Data are presented as mean (SD)

^a Oligoasthenozoospermia vs asthenozoospermia

^b Oligoasthenozoospermia vs controls

^c Asthenozoospermia vs controls

^d Fisher's exact test

level was higher in the sperm of asthenozoospermia patients than that of oligoasthenozoospermia patients. In contrast to the *CIB1* mRNA levels, we found that mRNA expression levels of the cell cycle regulator *CDK1* were elevated in oligoasthenozoospermia patients relative to controls ($P<0.01$, Fig. 1d–f), and the *CDK1* mRNA level was also higher in the sperm of oligoasthenozoospermia patients than in sperm from asthenozoospermia patients.

CIB1 protein expression patterns oppose those of *CDK1* in the sperm of oligoasthenozoospermia patients

Western blot analysis revealed that the protein levels of *CIB1* in sperm from control patients were higher than those in sperm from either asthenozoospermia patients or oligoasthenozoospermia patients (Fig. 2a). In addition, the protein levels of *CDK1* in sperm from control

Fig. 1 Expression of *CIB1* and *CDK1* mRNA in sperm from different groups. **a, b** RT-PCR analysis of *CIB1* expression. **c** Expression of *CIB1* as detected by real-time RT-PCR. **d, e** RT-PCR analysis of *CDK1* expression. **f** Expression of *CDK1* as detected by real-time RT-PCR. We used β -actin as an internal control. 1, Oligoasthenozoospermia, 2, Asthenozoospermia, 3, Control. ^s vs. oligoasthenozoospermia group; [#] vs. asthenozoospermia group. ^{ss} $P<0.01$; ^{##} $P<0.01$; ^{sss} $P<0.001$; ^{####} $P<0.001$

Fig. 2 Expression of CIB1 and CDK1 protein in sperm from different groups. Western blotting detection of CIB1 (a) and CDK1 (b) protein expression. Upper: Representative Western blotting showing the relevant bands. Lower: analysis of the relative intensity of CIB1 or CDK1 protein. We used β -actin as an internal control. 1, Oligoasthenozoospermia, 2,

Asthenozoospermia, 3, Control. $^{\$}$ vs. oligoasthenozoospermia group; $^{\#}$ vs. asthenozoospermia group. $^{\$\$}$ $P < 0.01$; $^{\$}$ $P < 0.05$; $^{\#}$ $P < 0.05$. Differences between three groups were determined by one-way ANOVA ($n = 5$ patients in each group)

patients were lower than those from patients with asthenozoospermia and oligoasthenozoospermia (Fig. 2b).

Correlation between *CIB1* expression and clinical features and *CDK1* expression

Linear regression analysis revealed a strong statistically significant negative correlation between *CIB1* expression and *CDK1* expression ($r^2 = +0.397$; $P < 0.001$); however, the correlations between *CIB1* expression and age, BMI, sexual abstinence time, total semen volume, sperm density, normal sperm morphology, and sperm viability were not statistically significant.

Discussion

CIB1 is a widely expressed protein and has been identified as an important regulatory Ca^{2+} -binding protein that is involved in various cellular functions. CIB1 binds to a number of potential effectors, such as PKD2 [18], AKT, ERK [19], PAK1 [20], PLK3 [21, 22], and the functions of some of these proteins have been shown to be influenced by CIB1. These studies suggest that CIB1 regulates tumor growth, cell migration, endomitosis, and cell cycle progression. Notwithstanding, the functions of endogenous CIB1 and its relationship to relevant intracellular binding partners have not been clearly delineated. Several studies have shown that CIB1 plays an important role in mouse spermatogenesis [13] and ram fecundity [14]. In our study, we found that *CIB1* mRNA expression levels were lower in sperm of oligoasthenozoospermia and asthenozoospermia patients than in controls, and that the corresponding protein expression levels were significantly lower in sperm of oligoasthenozoospermia and asthenozoospermia patients than in that of controls. In addition, we found

that the mRNA and protein expression levels of CDK1 were the opposite of that of CIB1 in the sperm of oligoasthenozoospermia and asthenozoospermia patients and controls, suggesting that the mRNA and protein expression levels of these genes can distinguish infertile patients from fertile controls. Such information will not only increase the number of novel biomarkers for molecular diagnostics in infertility, but may also indicate a molecular mechanism underlying the spermatogenetic processes that regulate and control fertility.

The molecular basis of male fertility dysfunction is largely unknown; however, several studies have revealed the role of certain genes or miRNAs in the male reproductive system [23, 24]. We observed lower expression levels of *CIB1* in oligoasthenozoospermia and asthenozoospermia patients than in control fertility patients. This result is intriguing, since CIB1 has previously been implicated in regulation of cell growth and division. CIB1 binds to cell cycle-regulatory proteins, such as PLK2, PLK3, and DNA-PK [21, 22, 25], and thus loss of CIB1 may lead to a delay in cell growth through one or more of these proteins. Moreover, we found that *CDK1* mRNA and protein levels significantly increased in the sperm of oligoasthenozoospermia patients, in contrast to those of *CIB1*, and our results showed that *CIB1* mRNA levels were significantly associated with *CDK1* mRNA levels in this study group. This increase in CDK1 protein expression in the sperm of oligoasthenozoospermia patients and asthenozoospermia patients may reflect a compensatory increase that is directly related to the loss of CIB1 in these patients. Thus, it is possible that CIB1 participates in the CDK1 signaling pathway (or vice versa) and that loss of CIB1 affects the cell cycle in germ cells.

In our previous study, we found that *CIB1* was widely expressed in different tissues in sheep, with the highest level found in the testes, suggesting that it may play a role in ram

fertility [14], while another family member, CIB4, was only detected in sheep testes, and the short spliced form of this transcript, *S-CIB4*, could only be detected in a breed of sheep with high fecundity [15]. Furthermore, the present study identified that there was an altered CIB1 profile in the sperm of infertile patients and fertile controls. These findings suggest an important role for CIB1 in male reproductive function, and an association may exist with human infertility. Our results showed that CDK1 protein levels increased in oligoasthenozoospermia patients and asthenozoospermia patients, with the highest level being reached in oligoasthenozoospermia patients. Changes in the expression of CDK1 or other cell cycle-regulators could disrupt the normal interval of Sertoli cell proliferation, causing an imbalance between the numbers of Sertoli cells and developing germ cells [26], which could lead to increased germ cell apoptosis and defective spermatogenesis [27]. Sertoli cells secrete a variety of endocrine and paracrine factors that regulate spermatogenesis [28]. Another possibility is that loss of CIB1 may lead to defects in Sertoli cell differentiation and function, which are required for the maintenance of spermatogenesis.

Our study has a number of limitations. The key limiting factor for testing mRNA and protein expression levels of *CIB1* and *CDK1* in this study was the lack of testes samples; thus, further investigations are required.

In conclusion, we have defined the profile of CIB1 in infertile patients and fertile controls, which may serve as an adjunct biomarker for diagnosing male infertility. Moreover, there was a negative correlation between *CIB1* and *CDK1* expression. These results yield further insights into the molecular mechanisms underlying male infertility and the clinical value of CIB1 in infertility.

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References

- Feng HL. Molecular biology of male infertility. *Arch Androl*. 2003;49(1):19–27.
- Skakkebaek NE, Jorgensen N, Main KM, Rajpert-De Meyts E, Leffers H, Andersson AM, et al. Is human fecundity declining? *Int J Androl*. 2006;29(1):2–11.
- Okada H, Tajima A, Shichiri K, Tanaka A, Tanaka K, Inoue I. Genome-wide expression of azoospermia testes demonstrates a specific profile and implicates ART3 in genetic susceptibility. *PLoS Genet*. 2008;4(2):e26.
- Dada R, Kumar M, Jesudasan R, Fernandez JL, Gosalvez J, Agarwal A. Epigenetics and its role in male infertility. *J Assist Reprod Genet*. 2012;29(3):213–23.
- Organization WH. WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction. 5th ed. Cambridge, United Kingdom: Cambridge University Press; 2010.
- Attia AM, Al-Inany HG, Farquhar C, Proctor M. Gonadotrophins for idiopathic male factor subfertility. *Cochrane Database Syst Rev*. 2007;4, CD005071.
- Colacurci N, Monti MG, Fornaro F, Izzo G, Izzo P, Trotta C, et al. Recombinant human FSH reduces sperm DNA fragmentation in men with idiopathic oligoasthenoteratozoospermia. *J Androl*. 2012;33(4):588–93.
- Publicover S, Harper CV, Barratt C. [Ca²⁺]_i signalling in sperm—making the most of what you’ve got. *Nat Cell Biol*. 2007;9(3):235–42.
- Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, et al. Ca²⁺ stores in sperm: their identities and functions. *Reproduction*. 2009;138(3):425–37.
- Espino J, Mediero M, Lozano GM, Bejarano I, Ortiz A, Garcia JF, et al. Reduced levels of intracellular calcium releasing in spermatozoa from asthenozoospermic patients. *Reprod Biol Endocrinol*. 2009;7:11.
- Naik MU, Naik UP. Calcium- and integrin-binding protein regulates focal adhesion kinase activity during platelet spreading on immobilized fibrinogen. *Blood*. 2003;102(10):3629–36.
- Kostyak JC, Naik MU, Naik UP. Calcium- and integrin-binding protein 1 regulates megakaryocyte ploidy, adhesion, and migration. *Blood*. 2012;119(3):838–46.
- Yuan W, Leisner TM, McFadden AW, Clark S, Hiller S, Maeda N, et al. CIB1 is essential for mouse spermatogenesis. *Mol Cell Biol*. 2006;26(22):8507–14.
- Yu Y, Song X, Du L, Wang C. Molecular characterization of the sheep CIB1 gene. *Mol Biol Rep*. 2009;36(7):1799–809.
- Yu Y, Zhang Y, Song X, Jin M, Guan Q, Zhang Q, et al. Alternative splicing and tissue expression of CIB4 gene in sheep testis. *Anim Reprod Sci*. 2010;120(1–4):1–9.
- Bliss SB, Voge JL, Hayden SS, Teague SR, Brinsko SP, Love CC, et al. The impact of cushioned centrifugation protocols on semen quality of stallions. *Theriogenology*. 2012;77(6):1232–9.
- Marone M, Mozzetti S, De Ritis D, Pierelli L, Scambia G. Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. *Biol Proced Online*. 2001;3:19–25.
- Armacki M, Joodi G, Nimmagadda SC, de Kimpe L, Pusapati GV, Vandoninck S, et al. A novel splice variant of calcium and integrin-binding protein 1 mediates protein kinase D2-stimulated tumour growth by regulating angiogenesis. *Oncogene*. 2013.
- Leisner TM, Moran C, Holly SP, Parise LV. CIB1 prevents nuclear GAPDH accumulation and non-apoptotic tumor cell death via AKT and ERK signaling. *Oncogene*. 2013;32(34):4017–27.
- Naik MU, Naik UP. Contra-regulation of calcium- and integrin-binding protein 1-induced cell migration on fibronectin by PAK1 and MAP kinase signaling. *J Cell Biochem*. 2011;112(11):3289–99.
- Naik MU, Naik UP. Calcium- and integrin-binding protein 1 regulates microtubule organization and centrosome segregation through polo like kinase 3 during cell cycle progression. *Int J Biochem Cell Biol*. 2011;43(1):120–9.
- Naik MU, Pham NT, Beebe K, Dai W, Naik UP. Calcium-dependent inhibition of polo-like kinase 3 activity by CIB1 in breast cancer cells. *Int J Cancer*. 2011;128(3):587–96.
- Qin Y, Han X, Peng Y, Shen R, Guo X, Cao L, et al. Genetic variants in epoxide hydrolases modify the risk of oligozoospermia and asthenospermia in Han-Chinese population. *Gene*. 2012;510(2):171–4.
- Wang C, Yang C, Chen X, Yao B, Zhu C, Li L, et al. Altered profile of seminal plasma microRNAs in the molecular diagnosis of male infertility. *Clin Chem*. 2011;57(12):1722–31.
- Koike M, Awaji T, Kataoka M, Tsujimoto G, Kartasova T, Koike A, et al. Differential subcellular localization of DNA-dependent protein

- kinase components Ku and DNA-PKcs during mitosis. *J Cell Sci.* 1999;112(Pt 22):4031–9.
26. O'Brien DA. Isolation, separation, and short-term culture of spermatogenic cells, p. In R. E. Chapin and J. J. Heindel (ed.), *Methods in toxicology*, vol. 3A. Academic Press, New York, N.Y.; 1993. p 246–64.
27. Urano A, Endoh M, Wada T, Morikawa Y, Itoh M, Kataoka Y, et al. Infertility with defective spermiogenesis in mice lacking AF5q31, the target of chromosomal translocation in human infant leukemia. *Mol Cell Biol.* 2005;25(15):6834–45.
28. Griswold MD. The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol.* 1998;9(4):411–6.