

Original Article

An insight into insulin-like factor 3 regulate its receptor RXFP2 in mouse gubernaculum testis cells

Shouxing Duan^{1,2,3}, Xuan Zhang⁴, Xuewu Jiang⁴, Lei Xie^{2,3}, Zongbo Sun^{2,3}, Shuhua Ma^{2,3}, Jianhong Li¹

¹Department of Pediatric Surgery, The Second Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong, China; ²Department of Radiology, The First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong, China; ³Guangdong Key Laboratory of Medical Molecular Imaging, Shantou, Guangdong, China; ⁴Department of Pediatric Surgery, The Affiliated Maternal and Child Health Hospital of Shenzhen University Medical College, Shenzhen, Guangdong, China

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Abstract: The etiology of testicular dysgenesis syndrome is multifactorial and involves abnormalities in the anatomical structures and endocrine factors. Several studies have shown that the abnormal development of the gubernaculum may affect testicular descent, and the insulin-like factor 3 (INSL3) appears to play an important role in development of the gubernaculum have been proved. INSL3 binds its specific receptor (Relaxin family peptide 2, RXFP2), which was highly expressed in gubernaculum, to produce a crucial effect in the first transabdominal descent stage, but its mechanism still remain unclear. In this study, in order to explore how does INSL3 regulate its receptor RXFP2, we cultured mouse gubernaculum testis cells in vitro, which was treated by INSL3, and examined the expression of RXFP2 in mouse gubernaculum testis cells. The results displayed that INSL3 changed RXFP2 expression, and we found that low dose INSL3 can increase RXFP2 expression, the mechanism of above-mentioned might be related with the hormesis of INSL3.

Keywords: INSL3, RXFP2, gubernaculum, mouse

Introduction

Cryptorchidism is the most common disorder of sexual differentiation in humans, with a 3.5% incidence in term newborns [1]. It can result in infertility and is associated with an increased risk of testicular cancer [2]. Cryptorchidism is the failure of the testis to descend into the scrotum, testicular descent is a complex process in testes development, and the gubernaculum plays an essential role in the complex mechanism of testicular descent [3].

Normal testicular descent has been described as two phases, transabdominal and inguinoscrotal migration phases. The migration involves the regression of the cranial suspensory ligament and the development of the gubernaculum. INSL3 has been proved to have a direct stimulatory effect on fetal gubernaculum growth, especially in transabdominal testicular descent [4]. RXFP2, previously known as Great or Lgr8, is the only receptor for INSL3 [5],

and the RXFP2 receptor is high expressed in the gubernaculum⁴. Bilateral cryptorchidism in INSL3- and RXFP2- deficient mice is due to impaired development of the gubernaculum [6, 7]. These findings clearly demonstrate the role of INSL3/RXFP2 signaling in the process of testicular descent and the gubernacular growth.

However, the mechanisms by which INSL3 and RXFP2 respectively modulate the development of gubernaculum testis cells and how they interact together remain elusive. The goal of the present study was to evaluate the influence of RXFP2 by its ligand INSL3, and explore the mechanism of testicular descent further.

Materials and methods

Primary cell culture

Kunming mice were mating that were maintained at the Center of Animal in the Medical College of Shantou University. After born, 3

Insulin-like factor 3 regulate RXFP2 in vitro

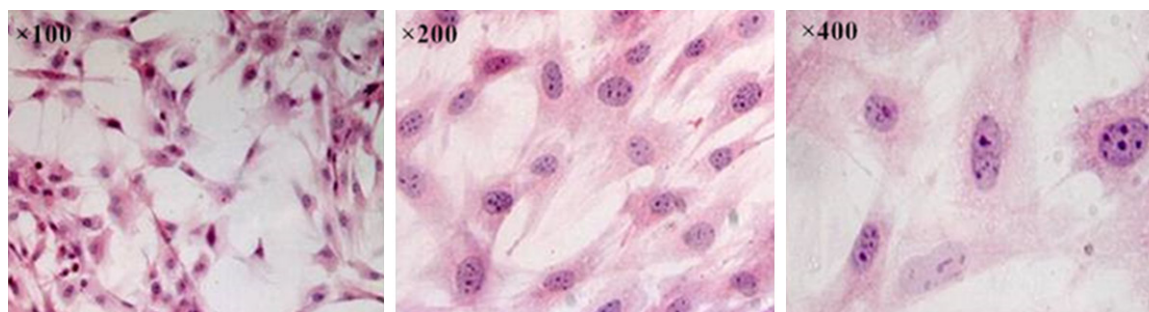


Figure 1. The staining of HE displayed the culture cells were fibroblast-like and highly homogeneous in morphology (scanning for $\times 100$, $\times 200$, $\times 400$ respectively).

Table 1. Expression of RXFP2 mRNA ($n = 3$, $\bar{x} \pm s$)

Groups	RC value (RT-PCR)
20 ng/ml	0.0425 \pm 0.0016
2 ng/ml	0.0441 \pm 0.0003
0.2 ng/ml	0.1020 \pm 0.0038
0.02 ng/ml	0.4581 \pm 0.0141*
Normal (nothing)	0.0596 \pm 0.0086

* $P < 0.01$ vs. group of normal. RC means relative coefficient = samples value/ β actin value.

days neonatal mice were killed by decapitation and its gubernaculum tissue was collected. The tissue were removed under an operating magnifier and placed into phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mg/ml type I collagenase for 1 hour. Cells were grown under 5% CO_2 and 95% air in phenol red-free DMEM containing 5% charcoal dextran-treated fetal bovine serum (FBS).

Treatment and grouping of INSL3

The subcultured cells were randomly divided into different groups treated with different concentrations of INSL3 (Phoenix, USA). The groups including normal (nothing), and experimental groups (treated INSL3 at 20, 2, 0.2, and 0.02 ng/ml, respectively). Treatment 48 hours, the gubernaculum cell for following experiments.

Semiquantitative reverse transcriptase PCR

Total RNA of gubernaculum cells were extracted by Trizol reagent (Invitrogen, USA) and quantified by UV spectrophotometry. The mRNA was reverse-transcribed to cDNA in a total volume of 20 μ l with random primers (Sangon,

Shanghai, China). The PCR program consisted of an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds, elongation at 72°C for 60 seconds, and a final elongation at 72°C for 5 minutes. β -actin was amplified simultaneously as internal control. The primers: RXFP2 upstream, 5' TACCTGTCTCCGTGGGCGTCTT 3', and downstream, 5' CCGATGTGGCTCCTCACTTCTGC 3' (amplification length, 494 bp) and β -actin upstream, 5' GAGACCTCAACACCCAGC 3', and downstream 5' CCACAGGATTCCATACCCAA 3' (amplification length, 446 bp). The primers were designed using the Primer Premier 5.0 software and were synthesized by Shanghai Sangon Biological Technology Co., Ltd. (Shanghai, China).

Flow cytometry analysis

Gubernaculum cells were collected after treatment 48 hours by INSL3, fixed with 70% alcohol at 4°C for overnight, washed in PBS and centrifugation sedimentation, Cells were incubated overnight at 4°C with a goat polyclonal antibody against mice RXFP2 (1:100, Santa Cruz, USA) followed by incubation with fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat IgG (1:50, Boster China) for 2 hour at indoor temperature. Cell suspension was detected by flow cytometry (BD, USA).

Statistical analysis

All of the data are expressed as the mean \pm standard deviation, The results were analyzed by an analysis of variance (ANOVA) test using SPSS 13.0 software. The bands from RT-PCR and the histogram from FCM were semi-quantified with BandScan 5.0 and WinMDI 2.9 soft-

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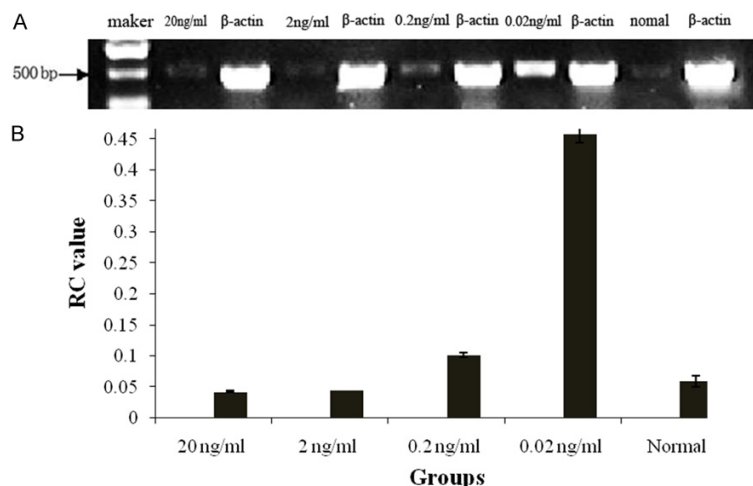


Figure 2. Expression of RXFP2 mRNA after treatment with different concentrations of INSL3 (A, B). RC means relative coefficient = samples value/beta actin value. The INSL3 groups were given 20 ng/ml, 2 ng/ml, 0.2 ng/ml, and 0.02 ng/ml respectively (* $P < 0.01$ vs. normal group).

Table 2. Expression of RXFP2 protein (n = 3, $\bar{x} \pm S$)

Groups	FI value (FCM)
20 ng/ml	21.9233 \pm 2.4567
2 ng/ml	25.4933 \pm 3.1160
0.2 ng/ml	24.6267 \pm 2.0067
0.02 ng/ml	86.5667 \pm 1.9912*
Normal (nothing)	21.3667 \pm 2.1000

* $P < 0.01$ vs. group of normal; FI means fluorescence index = fluorescence intensity of samples-fluorescence intensity of blank control.

ware, respectively. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Hematoxylin and eosin staining of gubernaculum cells

HE staining revealed that the cells were highly homogeneous in morphology. The gubernaculum cells were mainly fibroblasts-like, a confluent monolayer of fibroblasts formed which were interspersed with a few epithelioid cells (**Figure 1**).

Effect of INSL3 treatment on expression of RXFP2 mRNA

Compared with the normal group, the expression of RXFP2 mRNA in 0.02 ng/ml INSL3

group was upregulated ($P < 0.01$), and there were no significant difference in the 20 ng/ml, 2 ng/ml, 0.2 ng/ml groups ($P > 0.05$). (**Table 1; Figure 2A, 2B**).

Effects of INSL3 treatment on protein expression of RXFP2

The results showed that INSL3 changed the protein expression of intracellular RXFP2. Compared with the control group, there were no significant differences in the 20 ng/ml, 2 ng/ml, 0.2 ng/ml groups ($P > 0.05$), but the protein expression of RXFP2 in 0.02 ng/ml INSL3 group was upregulated significantly ($P < 0.01$) (**Table 2; Figure 3A, 3B**).

Discussion

Cryptorchidism is a common congenital anomaly of the urogenital tract in human males, it is the absence of one or both testes from the scrotum. The testes begin as an immigration of primordial germ cells into testicular cords along the gonadal ridge in the abdomen of the early embryo. It has been proposed that movement occurs in two phases, transabdominal and inguinoscrotal migration phases. The migration involves the regression of the cranial suspensory ligament and the development of the gubernaculum [8, 9]. Due to the special anatomic location of gubernaculum contiguous to the testes, gubernaculum is crucially involved in testicular descent and development [10, 11]. The etiology of cryptorchidism is multifactorial and might involve abnormalities in the anatomical structures and endocrine factors.

Previous studies have demonstrated that cryptorchidism may frequently be associated with mutations in the genes for INSL3 and its receptor RXFP2. These genes seem to have an important role in testicular descent without apparently affecting the spermatogenic and endocrine components of the testis itself, INSL3 and RXFP2 proteins seem to act as ligand and receptor respectively, and to play a prominent role in gubernaculum development involved in testicular descent [12, 13]. Insulin-like factor 3 (INSL3), a peptide belonging to the

Insulin-like factor 3 regulate RXFP2 in vitro

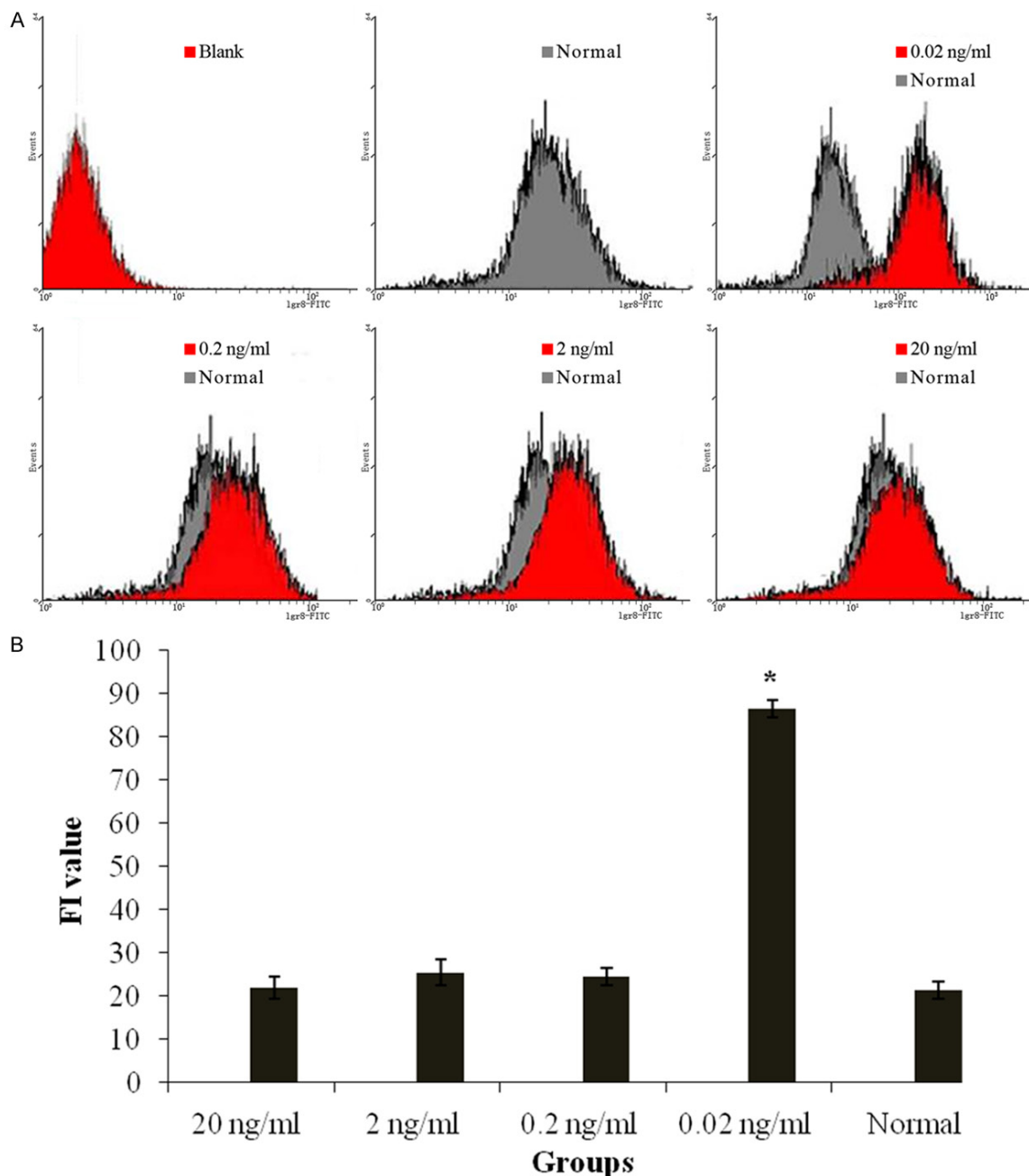


Figure 3. The red-tinted region and gray region represent the fluorescence intensity of samples and normal control respectively (A). Protein expression of RXFP2 after different concentrations INSL3 treated (B). FI means fluorescence index = fluorescence intensity of samples - fluorescence intensity of blank control. The INSL3 groups were given 20 ng/ml, 2 ng/ml, 0.2 ng/ml, and 0.02 ng/ml respectively (*P < 0.01 vs. normal group).

insulin-relaxin family and also known as Leydig cell-derived insulin-like factor [13]. Mice mutants for INSL3 exhibit bilateral cryptorchidism and developmental abnormalities of the gubernaculum [14]. Relaxin family peptide 2 (RXFP2), previously known as Great or Lgr8, ini-

tially discovered as a low-affinity relaxin receptor, has now been characterized as the INSL3 special receptor [15]. Similarly to INSL3, the disruption of the RXFP2 gene leads to a cryptorchid phenotype in RXFP2-mutant mice [7, 16]. INSL3 acts by binding to its specific receptor

Insulin-like factor 3 regulate RXFP2 in vitro

RXFP2, how to interact together? There are still no reports.

To investigate the effects of INSL3 regulate its receptor RXFP2, we cultured mice gubernaculum testis cells in vitro and treated by single factor (INSL3) to avoid the interference of other factors. In present study we selected multiple dosages of INSL3 from 0.02 to 20 ng/ml to observe the change of RXFP2 in mouse gubernaculum cells after treated by INSL3. The data demonstrated that there were no significant difference in the 20 ng/ml, 2 ng/ml, 0.2 ng/ml groups compared with the normal group ($P > 0.05$), but the expression of RXFP2 in 0.02 ng/ml INSL3 group was upregulated ($P < 0.01$). These results show that low dose INSL3 can increase RXFP2 expression, the mechanism of above-mentioned might be related with the hormesis of INSL3. Additionally, there are some studies reported SOX9 might play a significant role in regulating RXFP2 activity [17, 18], but its specific mechanism is unknown and need further study. Nevertheless, these low levels of INSL3 may suffice to mediate the specific RXFP2 signaling in target tissues.

The present study provided novel evidence that INSL3 have direct mediate RXFP2 in gubernaculum testis cells, we speculate the synergistic effects of low dose INSL3 to its receptor RXFP2 in mouse gubernaculum testis cells provide an insight into the role of INSL3 in the etiology of cryptorchidism.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jianhong Li, Department of Pediatric Surgery, The Second Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong, China. Tel: 86 754 88915793; Fax: 86 754 88346543; E-mail: jianhongli2013@163.com; Dr. Shuhua Ma, Department of Radiology, The First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong,

China. Tel: 86 754 87833333; Fax: 86 754 87833033; E-mail: shuhuama2014@126.com

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Insulin-like factor 3 regulate RXFP2 in vitro

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