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Diethylstilbestrol Regulates the Expression of LGR8 in Mouse Gubernaculum Testis Cells

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Hormonal effects on the gubernaculum can affect testicular descent. Diethylstilbestrol (DES) is a nonsteroidal synthetic estrogen that disrupts the outgrowth of gubernaculums, leading to testis maldescent. However, the underlying mechanisms remain elusive.

Material/Methods: The gubernaculum were removed from 3-day-old mice and cultured. The subcultured cells were randomly divided into a normal control group and experimental groups. The DES groups were administered 10 μg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml of diethylstilbestrol dissolved in dimethyl sulfoxide (DMSO) respectively. The cell morphology was observed under an inverted microscope, and leucine-rich repeat-containing G protein-coupled receptor 8 (LGR8) was localized by immunofluorescence. The expressions of LGR8 gene and protein in gubernaculum cells were quantified by RT-PCR and Flow Cytometer respectively.

Results: DES treatment converted cells from a normal fibroblast-like morphology into a more refractile, spindle-shaped morphology or irregular elliptical shapes along with cytoplasmic shrinkage. LGR8 was expressed in the cytoplasmic membrane, DES dose-dependently downregulated LGR8 expression at low doses (≤1.0 μg/ml), but upregulated LGR8 at high doses (10 μg/ml) at both the mRNA and protein levels.

Conclusions: These results suggest that DES causes testicular maldescent by altering the LGR8 pathway in mouse gubernaculum testis cells.

MeSH Keywords: Diethylstilbestrol • Mice • Testicular Diseases

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Background

In recent years, many studies have shown that the male reproductive system is affected by environmental estrogens (EEs) [1,2]. EEs have been found in various pesticides, plastic products, flame retardants, and many other products that are needed for daily use [3]. Exposure to EEs leads to a high rate of reproductive abnormalities in males, such as hypospadia, low semen quality, testicular hypoplasia, and cryptorchidism. Cryptorchidism is one of the most common clinical manifestations. The incidence of testicular cryptorchidism is increasing worldwide. This increase has been theorized to be related to exposure to environmental estrogens [4]. Cryptorchidism is the failure of the testis to descend into the scrotum, affecting approximately 3% of full-term male infants, and can result in infertility. In addition, cryptorchidism is also associated with an increased risk for testicular torsion and development of testicular cancer [5,6]. However the etiology of cryptorchidism is multifactorial and still unclear.

Normal testicular descent has been described as involving two phases, the transabdominal and inguinoscrotal migration phases. During the transabdominal phase, the testis migrates from the urogenital ridge to the inguinal region, which occurs at 10–15 weeks in the embryo of humans and from embryonic day 15.5 (E15.5) to E19 in mice [6,7]. Numerous reports also showed that transabdominal testicular descent abnormalities in rodent fetuses are induced by exposure to DES [8,9], and the INSL3/LGR8 system is considered an important pathway in transabdominal testicular descent [10,11].

It well known that the gubernaculum is closely involved in testicular descent [12,13]. Insulin-like factor 3 (INSL3) participates in the outgrowth of the gubernaculum testis by binding to its only receptor, the leucine-rich repeat-containing G protein-coupled receptor 8 (LGR8), to cause testicular descent [14–17]. Currently, the effects of EEs on INSL3 have been extensively reported [7,9,10,14], but the effects of EEs on LGR8 are unknown. In this study, we cultured mouse gubernaculum testis cells *in vitro*, and treated them with DES, a prototype estrogen, and then we detected the expression of LGR8 in mouse gubernaculum testis cells to investigate the mechanism of testicular descent.

Material and Methods

Primary cell culture and treatment

Kunming mice were maintained at the Animal Center of the Medical College of Shantou University. Three-day-old neonatal mice were killed by decapitation and gubernaculum tissue was 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 h. Then it was placed in and grown under 5% CO_2 and 95% air in phenol red-free DMEM containing 5% charcoal dextran-treated fetal bovine serum (FBS). After 3 days, primary cells were harvested by trypsinization and transferred into each well of a 6-well culture plate. Subcultured cells were randomly divided into different groups including normal group (untreated), control group (treated with DMSO), and experimental groups (treated with different concentrations of DES at 0.01, 0.10, 1.00, and 10.00 μg/ml, respectively). At 48 hours following DES addition, gubernaculum cell morphology was observed under an inverted microscope.

Immunofluorescence for LGR8

Gubernacular cell monolayers were washed in phosphate-buffered saline (PBS), fixed with 4% formaldehyde in PBS for 15 min, and then blocked for 1 h in 1% bovine serum albumin (BSA) in PBS. Cells were incubated overnight at 4°C with goat polyclonal antibody against mouse leucine-rich repeat-containing G protein-coupled receptor 8 (LGR8; 1:100, Santa Cruz, USA) followed by incubation with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG (1:50, Boster, China) for 1 h. Cells were washed in PBS, sealed in glycerin, and visualized by conventional immunofluorescence with a fluorescence microscope (Leica, Germany).

Semiquantitative reverse transcriptase PCR

Total gubernacular cells RNA was extracted with Trizol reagent (Invitrogen, USA) and quantified by UV spectrophotometry. The mRNA was reverse-transcribed to cDNA using random primers in a total volume of 20 μl (Sangon, China). The PCR program consisted of an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s, elongation at 72°C for 60 s, and a final elongation at 72°C for 5 min. β-actin was amplified simultaneously as the internal control. The primers and PCR products for each gene are listed in Table 1.

Flow cytometry analysis

Gubernacular cells were collected after a 48-h treatment with DES, fixed with 70% alcohol at 4°C overnight, washed in PBS and subjected to centrifugation. Cells were incubated overnight at 4°C with goat polyclonal anti-murine LGR8 (1:100, Santa Cruz, USA), followed by incubation with fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat IgG (1:50, Boster China) for 2 h at room temperature. Cell suspensions were detected by flow cytometry (BD, USA).

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Table 1. Primes and PCR products for RT-PCR.

Genes	Primers	Products
β-actin	F: 5' GAGACCTTCAACACCCCAGC 3'	446 bp
	R: 5' CCACAGGATTCCATACCCAA 3'	
LGR8	F: 5' TACCTGTTCTCCGTGGGCGTCTT 3'	494 bp
	R: 5' CCGATGTGGCTCCTCACTTCTGC 3'	

F – forward; R – reverse.

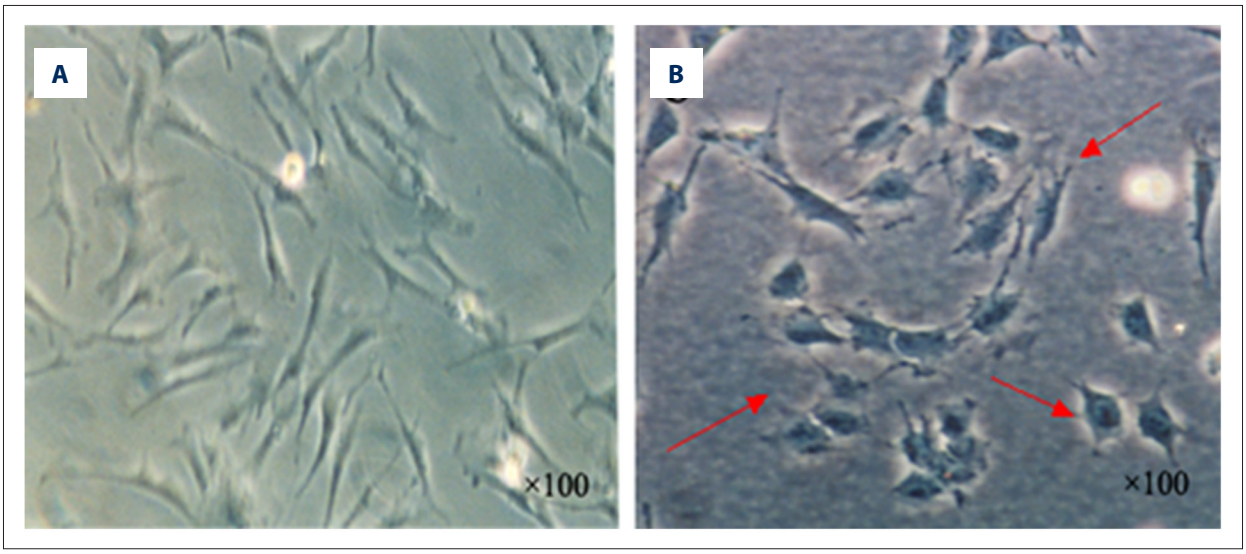

Figure 1. Cellular morphology under an inverted microscope: normal fibroblast-like cells take the shape of spindles or irregular triangles (**A** normal 48 h ×100); cells treated with 10 μg/ml DES show loss of fibroblast morphology, cytoplasmic shrinkage and irregular elliptic shapes (**B** DES 10 μg/ml 48 h ×100)

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). The results were analyzed by analysis of variance (ANOVA) using SPSS 13.0 software. Differences were considered significant at $P<0.05$. The bands from RT-PCR and the histogram from FCM were semi-quantified with BandScan 5.0 and WinMDI 2.9 software, respectively.

Results

Gubernacular cell morphology and cellular localization of LGR8

The cultured murine gubernacular cells were mainly fibroblasts-like, with confluent monolayers of fibroblasts being interspersed with a few epithelioid cells. Passaging of cells resulted in cells with highly homogeneous morphology and viability (Figure 1A). A 10-μg/ml dose of DES resulted in loss of fibrous morphology, and caused cytoplasmic shrinkage and an irregular elliptical shape (Figure 1B). LGR8 was highly expressed in the cell membrane, as judged by immunofluorescence and the fluorescence intensity of 10 μg/ml DSE group was higher (Figure 2).

Effect of DES treatment on expression of LGR8 mRNA

Compared with the normal group, the expression of LGR8 mRNA in the 10-μg/ml DES group was upregulated ($P<0.01$), but it was reduced in the 1-μg/ml, 0.1-μg/ml, and 0.01-μg/ml groups ($P<0.01$). There was no significant difference in the DMSO group ($P>0.05$) (Figure 3A, 3B; Table 2).

Effect of DES treatment on protein expression of LGR8

Addition of DES altered the protein expression of intracellular LGR8. Compared with the control group, the protein expression of LGR8 in the 10-μg/ml DES group was upregulated significantly ($P<0.01$), but it was reduced in the 1-μg/ml, 0.1-g/ml, and 0.01-μg/ml groups ($P<0.01$ or $P<0.05$). There was no significant difference in the DMSO group ($P>0.05$) (Figure 4A, 4B; Table 3).

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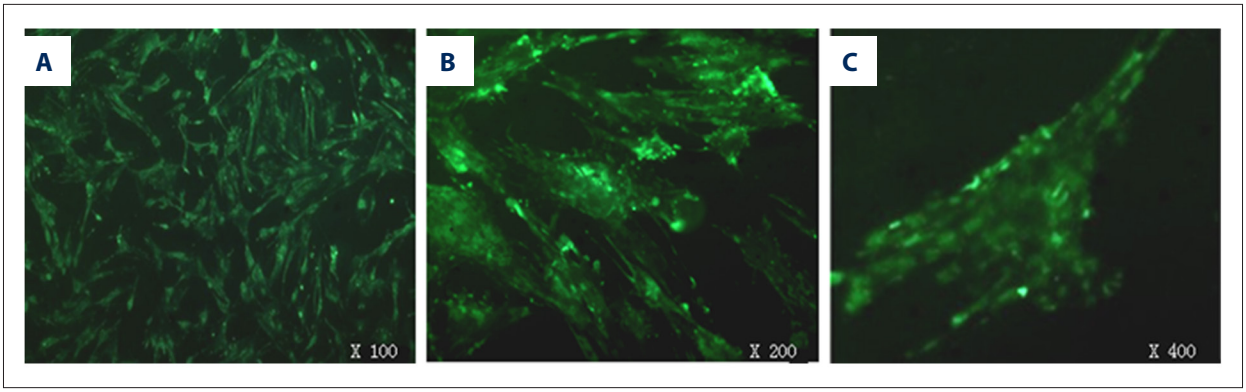

Figure. 2. LGR8 is highly expressed in the cell membrane. (**A** normal ×100; **B** normal ×200; **C** normal ×400).

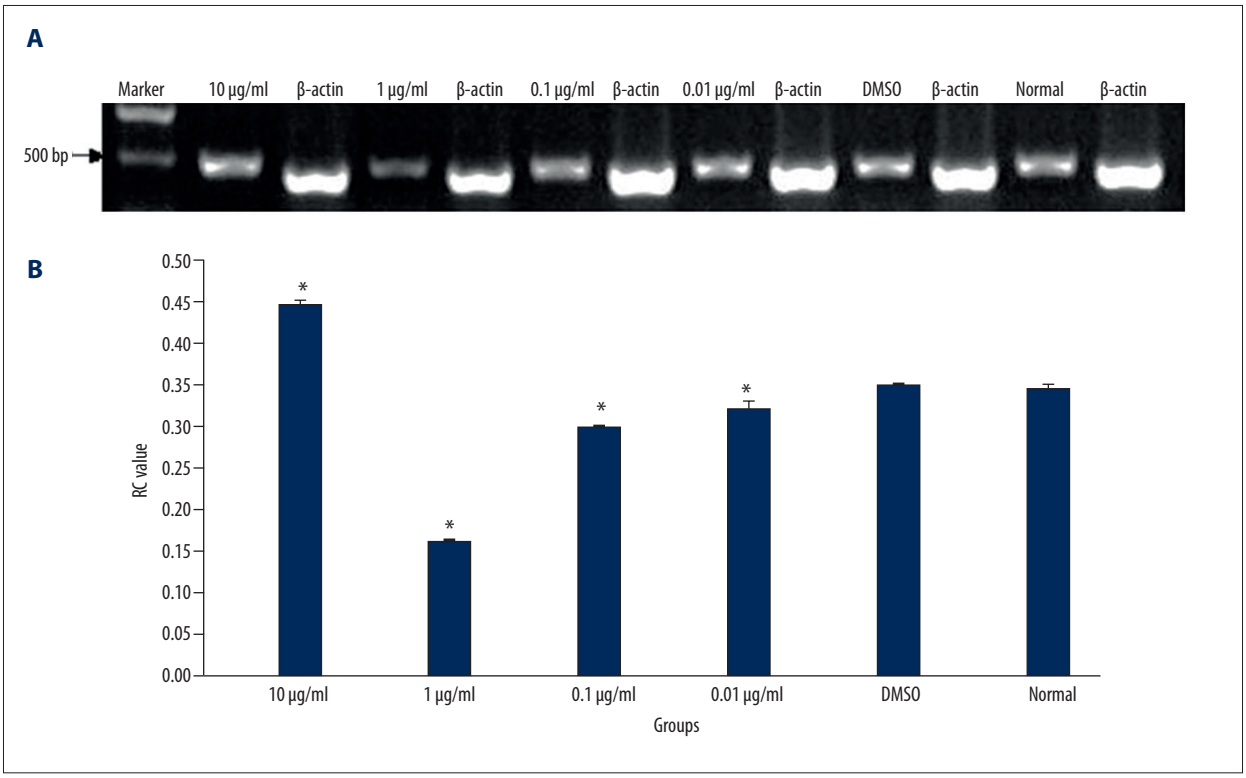

Figure 3. Expression of LGR8 mRNA after treatment with different concentrations of DES (**A, B**). The DES groups were given 10 μg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml, and dissolved in dimethyl sulfoxide (DMSO).

Table 2. The effect of different concentrations DES on the expression of LGR8 mRNA. (n=3, $\bar{\chi}$±s).

Groups	RC value (RT-PCR)
10 μg/ml	0.4481±0.0040*
1 μg/ml	0.1608±0.0020*
0.1 μg/ml	0.3005±0.0020*
0.01 μg/ml	0.3225±0.0040*
DMSO (control)	0.3527±0.0021
Normal (untreated)	0.3461±0.0043

* P<0.01 *vs.* normal group.

Discussion

The gubernaculum plays an essential role in the complex mechanism of testicular descent [12,13,17,18]. Insulin-like factor 3 (INSL3) appears to play an important role in testicular descent, which involves development of the gubernaculums [7,14,19]. In INSL3$^{-/-}$ mouse models, the bilateral testicular descent appears after INSL3 has been knocked out, but the gubernaculum testis will be small and poorly differentiated, with no mesenchymal cells in the central zone [10,20]. Several studies have shown that INSL3 is decreased by exogenous estrogen, such as diethylstilbestrol, 17 alpha-estradiol,

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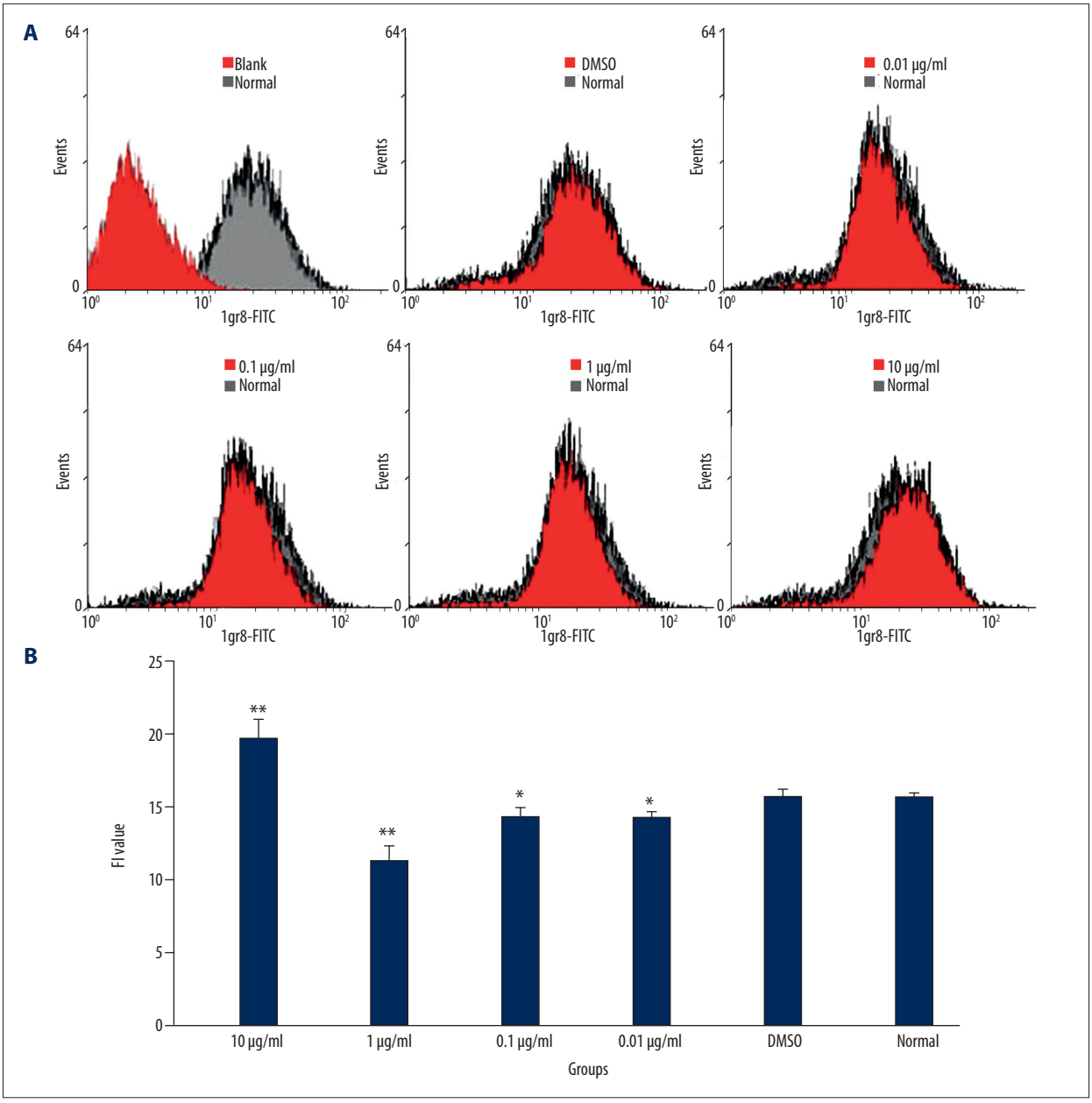

Figure 4. Protein expression of LGR8 after different concentrations DES treated (**A, B**). The DES groups were given 10 μg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml, and dissolved in dimethyl sulfoxide (DMSO)

Table 3. Expression of LGR8 protein. (n=3, $\bar{\chi}$±s).

Groups	FI value (FCM)
10 μg/ml	19.8457±1.1561**
1 μg/ml	11.4557±0.8475**
0.1 μg/ml	14.3890±0.5190*
0.01 μg/ml	14.3283±0.3218*
DMSO (control)	15.7257±0.5124
Normal (untreated)	15.7023±0.2593

** P<0.01 and * P<0.05 *vs.* normal group.

and 17 beta-estradiol [9,21,22]. INSL3 binds to its specific receptor (LGR8), which is highly expressed in the gubernaculum, to produce a crucial effect in the first transabdominal descent stage [17]. The LGR8 knockout phenotype is similar to the INSL3 knockout phenotype, indicating that INSL3 and LGR8 cannot work alone [11,16,23]. Therefore, LGR8 may be an important factor in testicular descent, but its mechanism remains unclear. Many experiments have focused on INSL3, but studies on LGR8 have rarely been reported.

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To investigate the effects on LGR8 by administering various doses of DES, we cultured mouse gubernaculum testis cells *in vitro* and treated them with a single factor (DES) to avoid the interference of other factors *in vivo*, and measured the expression of LGR8 in gubernaculum testis cells. In this study, we selected multiple dosages of DES, from 0.01 to 10.0 μg/ml, to observe the expressions of LGR8 mRNA and protein in mouse gubernacular cells. We observed that alterations in the expression of LGR8 at the protein level are consistent with changes in LGR8 mRNA expression. Compared with the normal group, the experimental groups were significantly different ($P<0.05$ or $P<0.01$), but the control group was not significantly different ($P>0.05$). These results confirm that exposure to DES in murine gubernaculum testis cells leads to a dose-dependent decrease in the expression of LGR8 mRNA and protein at low doses, but LGR8 mRNA and protein increased at a DES dose of 10.0 μg/ml.

In the present study, we found the morphology of cells is significantly changed at a DES dose of 10 μg/ml, with cells displaying outgrowth retraction, accumulation of intracellular particles, and loss of basic fibroblast morphology. These results suggest that 10 μg/ml maybe the most effective concentration at which to affect the mouse gubernaculum testis cells by DES *in vitro*. This corresponds with the phenomenon that exposure to a dose of estrogen in pregnancy may lead to fetal reproductive system malformation. The mechanism by which DES up-regulates LGR8 may be correlated with Sry-related high mobility group box 9 (SOX9). Earlier research reported SOX9 might play a specific role in regulating LGR8 activity, but its specific mechanism remains unclear and needs further study [6,24,25]. In our study, we proved that DES has a direct effect on LGR8 expression in gubernaculum testis cells, and provide a new insight that DES perturbs the growth of gubernaculum or testicular descent, perhaps through the LGR8 pathway, not just by INSL3.

Conclusions

In conclusion, we found that DES has a direct effect on LGR8 expression in gubernaculum testis cells. In addition, we previously showed that the effects of DES on the gubernaculum testis are generally direct [21,25]. Therefore, it is reasonable to hypothesize that the effects of DES on mouse gubernaculums testis cells may be mediated directly or indirectly by the LGR8 pathway, which can be further studied using DES to affect the development of the gubernaculum testis.

Declaration of interest

The authors declare that they have no competing interests.

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