

Insulin 3-Like Hormone and its Role in Epididymo-Testicular Descent

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ABSTRACT

Purpose: The role of insulin 3-like (Insl3) hormone signaling in the testicular descent process has been demonstrated. The purpose of the present study was to evaluate epididymal development in Insl3-deficient mice.

Materials and Methods: Heterozygous and homozygous Insl3 mutants of a mixed CD1 X 129/Sv genetic background were generated by breeding Insl3^{-/-} females with Insl3^{+/-} males, and their genotypes were determined by polymerase chain reaction. On the first postnatal day, newborn males were sacrificed, embedded in paraffin, and cut in 4 µm sections. Sections were stained with hematoxylin/eosin and immunoreacted with anti-α actin antibody.

Results: An analysis of stained sections indicated an arrest in the development of the epididymis in all homozygous mice. The cauda and corpus of the epididymis were undersized. Compared to the heterozygous epididymis, the homozygous epididymis had fewer peritubular layers and dwarfish musculature. We confirmed this with immunostaining with monoclonal antibodies against α-smooth muscle actin.

Conclusion: Defective development of the smooth musculature in the epididymis of Insl3 homozygous mutant mice, combined with its high intraabdominal undescended position, supports previous observations regarding the importance of intact epididymis morphology and function for descent of the epididymo-testicular unit.

Key words: cryptorchidism; epididymis; Insl3; mice, mutant strains

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INTRODUCTION

During early embryonic development of the urogenital tract, mesentery connects the gonads and the Wolffian and Müllerian ducts to the abdominal wall. During male and female development, two parts of the genital mesentery, the cranial suspensory ligament and the caudal genital ligament gubernaculum, are believed to be responsible for sexual dimorphism in the position of testis and ovary (1). It was reported that defects in this developmental process could cause cryptorchidism. Cryptorchidism is the most common

disorder of sexual differentiation in humans, with a 3.5% incidence in term newborns (2).

The mechanism controlling testicular descent in mice was determined by analyzing mouse lines that lack insulin-like 3 (Insl3) hormone and its receptor, Lgr8/Great (3). Bilateral cryptorchidism in Insl3- and Lgr8- deficient mice is due to impaired development of the gubernaculum (4,5). These findings clearly demonstrate the role of Insl3 signaling in the process of testicular descent.

Restricted expression of Insl3 in pre- and postnatal Leydig cells is mediated by steroidogenic

factor 1 (SF1). Insl3 secretion is dependent on the differentiating effect of leutinizing hormone (LH) on Leydig cells and is independent of steroidogenic LH-mediated action (6-8). In Insl3- deficient mice, the development of epididymis that descends with the testis was considered to be normal and had no influence on descent of the epididymo-testicular unit (5). The goal of the present study was to evaluate epididymal development in Insl3- deficient mice and determine whether epididymal development is involved in the process of testicular descent.

MATERIALS AND METHODS

Heterozygous and homozygous Insl3 mutants of mixed CD1 X 129/Sv genetic background were generated by breeding Insl3^{-/-} females with Insl3^{+/-} males, and their genotypes were determined by polymerase chain reaction (4). On the first postnatal day, newborn males were euthanized with carbon dioxide gas, fixed with Bouin's fixative, and embedded in paraplast. To evaluate epididymal development in Insl3- deficient mice, 5 Insl3 homozygous and 3 Insl3 heterozygous mice were sectioned at 4 μ m. One homozygous mouse was cut in the frontal plane, and the remaining four homozygous and three heterozygous mice were cut in the sag-

ittal plane. Serial sections were stained with hematoxylin and eosin and examined under a light microscope.

For immunohistologic analysis, selected sections were mounted on slides. After deparaffinization, sections were treated with a 3% hydrogen peroxide/methanol solution to block endogenous peroxidase. Sections were preincubated for 1 h with 5% normal goat serum in 0.05% Triton X-100- phosphate buffered saline (PBS) and incubated overnight at 4°C in 1:200 diluted monoclonal anti- α actin antibody (Dako). Sections were washed with PBS and incubated with peroxidase-conjugated goat anti-mouse antibody at a 1:500 dilution (Dako) for 1 h at room temperature. After washing with PBS, immunoreactivity was detected by incubating the sections in a solution containing 3,3'-diaminobenzidine tetrahydrochloride.

RESULTS

The testes of all homozygous mice were localized either in a high intraabdominal position in proximity to the kidney (7/10) or in a transversal ectopic position, adjacent to the contralateral partner. In contrast, all 6 testes from heterozygous mice were located at the bladder neck (Figure-1). The gubernacular bulb (scrotal attachment) was less developed in homozygous mice compared to heterozy-

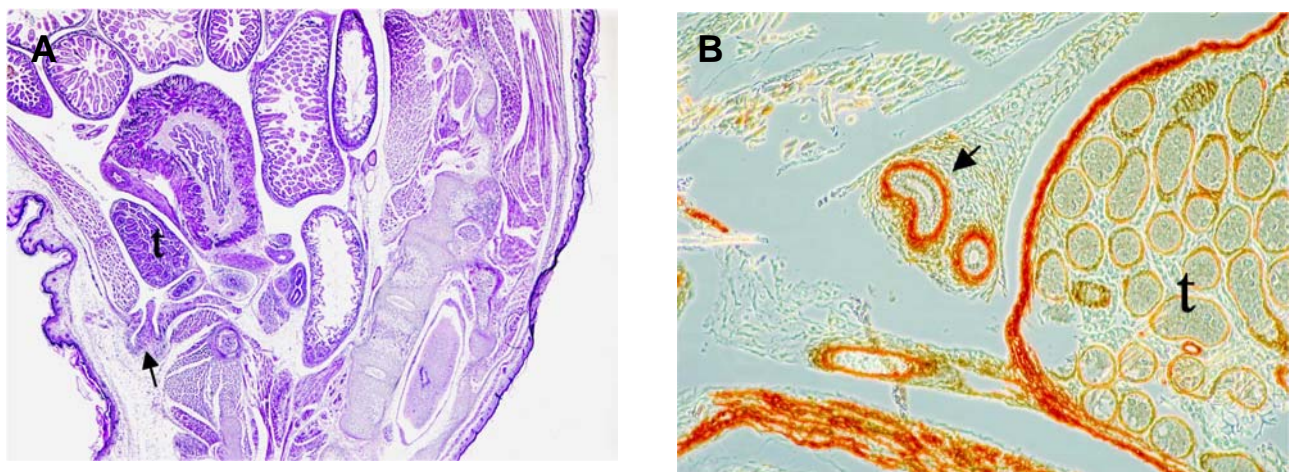


Figure 1 – A) Sagittal section of a heterozygous Insl3 mouse. The testis (t) is located at the bladder neck with developed cauda epididymis. The arrow points towards the gubernacular bulb, where the tip of the gubernaculum inserts. B) Immunostaining of the cauda epididymis showing strongly stained smooth muscle arranged in a circular fashion around the epididymal duct (arrow). The tunica albuginea and testicular peritubular connective tissue are also stained.

gous mice (Figure-2). In all homozygous mice, epididymis development was arrested. Both the cauda and corpus of the epididymis were severely under-sized (Figure-2). Compared to heterozygous mice, the epididymis of the homozygous mice had fewer peritubular layers and displayed a dwarfish musculature. Immunohistological staining for α -smooth muscle actin confirmed this (Figure-2). Immunostaining was absent in the testis and epididymal peritubular muscle layers of $Ins13^{-/-}$ mice, while it was strongly expressed in $Ins13^{+/-}$ mice (Figure-1). Interestingly, blood vessels stained intensely for α -smooth muscle actin in $Ins13^{+/-}$ and $Ins13^{-/-}$ mice, indicating a specific role of $Ins13$ in myogenesis of peritubular epididymal muscle layers (Figure-2). This

novel observation indicates that $Ins13$ signaling is involved in regulating the development of smooth muscle of the epididymis and testis.

COMMENTS

The role of the epididymis for descent of the epididymo-testicular unit has been postulated previously in experimental animals and human (9-11). August-Copenhagen-Irish rats have a congenital defect that frequently causes arrested development of a single ipsilateral Wolffian duct (9). If the Wolffian duct fails to form in the early developmental stage, the testis does not descend (9-11).

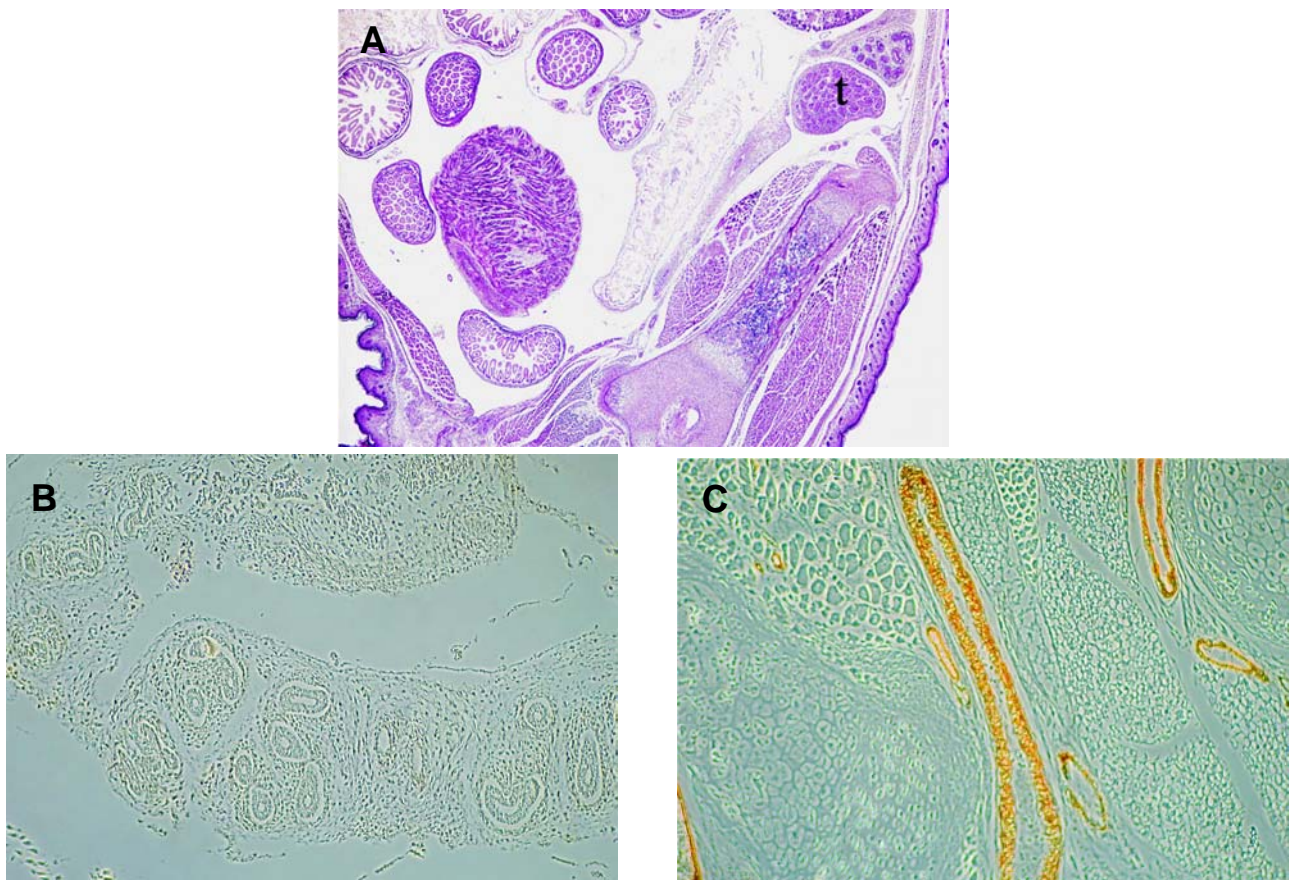


Figure 2 – A) Sagittal section of a homozygous $Ins13$ male mutant mouse with epididymis and testis (t) located in proximity to the lower kidney pole. The cauda and corpus of the epididymis are underdeveloped as is the gubernaculum region. B) Smooth musculature is absent around the epididymal duct in $Ins13$ homozygous male mutant mice. C) Vessels stained for monoclonal antibodies to smooth muscle actin in homozygous mice.

Treatment with LH-releasing hormone induces epididymo-testicular descent in 60% of naturally cryptorchid mice; and in these mice, increased testosterone secretion normalized the underdeveloped cryptorchid epididymis (10).

In 1984, Frey & Rajfer (12) reported that the distal gubernaculum is an absolute prerequisite for testicular descent, and they attempted to prove this by dissecting the distal part of the gubernaculum, repeating the experiment of Bergh et al. (13). However, shortly before birth, the rodent scrotum is partially inverted in the abdomen, giving an impression of being a part of the distal gubernaculum. It is self-evident that if the scrotum is stunted in its development, as Bergh et al. (13) showed, then the descent cannot take place. In contrast, scrotal development is normal in the vast majority of common cryptorchidism cases.

Since the gubernaculum in cryptorchid boys regresses after birth, the crucial question is why hormone treatment induces testicular descent, even though there is no gubernaculum in cryptorchid boys. In a placebo-controlled study (14) complete epididymo-testicular descent was achieved in cryptorchid boys who received hormone treatment. Hormone treatment induced a significant increase in serum testosterone (14). Boys with successful descent of the epididymis and testis had a normal-sized epididymis, while the majority of non-responders and surgically treated cryptorchid boys had small, irregular epididymes (15). Appropriate hormone treatment was capable of inducing increased testosterone secretion to stimulate further development of the epididymis and induce its descent into the scrotum (14,15). Our observation of defective development of the smooth musculature in the epididymis in *Insl3* homozygous mutant mice, combined with its high intraabdominal undescended position, supports previous observations regarding the importance of intact epididymis morphology and function for descent of the epididymo-testicular unit. Finally, the fact that there are animals with descended epididymes (chinchilla) but undescended testes, but no mammals with descended testes and undescended epididymes, underscores the necessity of epididymal, rather than testicular, descent (16).

CONFLICT OF INTEREST

None declared.

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EDITORIAL COMMENT

Although the authors have written to be focused on clarifying the controversy about the role of *Insl3* on the development of epididymis, they go further and stress the importance of epididymis for the descent of a testis. However, the epididymis is not mandatory for the descent (1,2). Contrary to the stated in the paper, the ACI rats have descended testes (3,4).

Their observations revealed a defective myogenesis in testis, epididymis and gubernaculum and supported the role of *Insl3* for myogenesis in special structures (5,6).

Their observation of defective myogenesis, together with the controversial role of epididymis, does not support their final conclusion about the importance of *insl3* for the descent of epididymo-testicular unit, and how does the defective myogenesis affect the descent remain obscure in their conclusion.

Since *Insl3* has a role in the myogenesis, and one of the current explanations of descent involves propulsion by the smooth muscle that depends on

myogenesis in the gubernaculum (7), their observation of defective myogenesis in the gubernaculum does not support the epididymal descent as they conclude, but seems to support the place of failed propulsion resulting from defective myogenesis.

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EDITORIAL COMMENT

According to the current view, normal testicular descent occurs in two phases. The first trans-abdominal phase (until week 15) is mainly depended on insulin-like peptide 3 (INSL3). The second inguinoscrotal phase is completed by week 35 and it is mainly depended on androgen action (1). In addition, androgens are of crucial importance for the development of the Wolffian duct derived organs, p.ex. epididymis. Testicular descent is conducted by gubernaculum, which is attached to the epididymis, which in turn is attached to the testis. Usually cryptorchidism is associated to the defective growth of the gubernaculum. However, epididymis is also important for testicular descent. This becomes evident also in some clinical situations. Sometimes the tip of the epididymis has grown to the scrotum together with the gubernaculum, while the other end of the elongated epididymis is lying in the abdominal cavity with the

testis. About one third of the cryptorchid testes are associated with some degree of epididymal abnormalities. In the present study, it is suggested that defective INSL3 action in addition to defective androgen action may cause abnormalities at least to the epididymal smooth musculature. It is unclear if epididymal abnormalities in general or the smooth muscle abnormalities detected in this study have some adverse effect for the fertility. Certainly, the surgeon has to be careful not to divide abnormal epididymis instead of gubernaculum during the operation.

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REPLY BY THE AUTHORS

The two stages of testicular descent are an old story presented by Gier & Marion in 1969 and 1970 (1,2) a long time before John Hutson even began his research. Furthermore, he published that the first phase of testicular descent is under the control of Mullerian Inhibiting Substance (MIS); this assertion is not true. Regarding INSL3 role in epididymo-testicular descent he should not be given credit for INSL3 only because he wrote about it in a review article. It is not his original discovery.

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