Asian Journal of Surgery (2019) 42, 290–296

Available online at www.sciencedirect.com

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journal homepage: www.e-asianjournalsurgery.com

ORIGINAL ARTICLE

Detection of insulin-like growth factor receptor-1 in the human cremaster muscle and its role in the etiology of the undescended testis

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Received 12 January 2018; received in revised form 13 February 2018; accepted 22 February 2018
Available online 31 March 2018

KEYWORDS
Undescended testis;
Cremaster muscle;
Insulin-like-growth factor receptor I

Summary *Purpose:* Previous studies of undescended testis (UT) has focused on insulin-like hormone 3 (INSL3), the genitofemoral nerve, and androgens in the testicular descent. Leydig cells, which are under the control of insulin-like growth factor-1 (IGF1), produce both androgens and INSL3. We aimed to investigate whether insulin-like growth factor receptor-1(IGFR1) exists in the cremaster muscle (CM) complex and is associated with normally descended testis as well as UT cases in humans.
Methods: We studied 30 CM from 15 patients who comprised the UT group (UTG), and 15 patients with unilateral testicular torsion (Control group; CG). Muscles, nerves, and vessels within the CM specimen were examined to determine the presence of IGFR1.
Results: The mean staining score (MSS) of IGFR1 in CM and its nerves were higher in the CG than in the UTG. These results were statistically significant ($p = 0.01$ and $p = 0.02$). Although the MSS of IGF1R was higher in the vessels of CM in the CG than the UTG, this was not statistically significant ($p = 0.48$).
Conclusions: IGFR1 with heterotetrameric receptor via IGF1, IGF2, insulin, and probably androgen, contribute to the remodeling and development of CM as well as the testis descent.

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https://doi.org/10.1016/j.asjsur.2018.02.005

In the current study, the presence of the IGFR1 in the CM was shown. Additionally, the IGFR1 density of the CM was lower in the UT cases than in the CG cases. Further evaluation of IGFR1 and other etiological factors can elucidate how they interact.

1. Introduction

Adrenogonadal development involving adrenal specification, testicular differentiation, and ovarian development occur as a complex differentiation. Using rodent models, insulin receptor (INSR) and the insulin-like growth factor receptor-1 (IGFR1) have been proven necessary to this development process. INSR and IGF1R null mice embryos have displayed reduced proliferation rates of somatic progenitor cells in both XX and XY gonads before sex determination, as well as complete agenesis of the adrenal gland and absence of testis development due to a reduction in splicing factor 1 (Sf1) gene expression and a failure of sex-determining region Y (Sry) upregulation.[1,2] IGFs have been shown to be the most important growth factor in regulating Sertoli cell (SC) number and testis size.[3] Fetal Leydig cells (LCs) produce both androgens and insulin-like hormone 3 (INSL3), which are responsible for the masculinization of the urogenital system and testicular descent.[4]

Previous reports have shown that IGF1 is implicated in LC development and function. According to these reports, testosterone production has been decreased by more than 80% in Igf1-null mice, and this deficiency is associated with a notable developmental delay in LCs and altered luteinizing hormone (LH)-stimulated androgen secretion in vitro. More reductions have also been observed in the expression of steroidogenic markers in the testis, such as StAR, Cyp11a1, and Cyp17a1.[5–7] Furthermore, IGFs are believed to be crucial for follicular maturation because they stimulate cell proliferation and steroidogenesis in the granulosa cells of different female species.[8]

The descent of the testis has been reported to occur in two stages: In the first stage, the transabdominal phase, which occurs between 8 and 15 weeks of gestation in humans, the testis descends to the internal inguinal ring by the enlargement of the caudal ligament named the gubernaculum.[9] In animal studies, the male-like development of the gubernaculum is dependent on the INSL3 and its receptor leucine-rich repeat-containing G protein-coupled receptor 8 (LGR8).[10,11] Previous research has investigated whether a growth stimulator or factor is required for fetal LCs producing INSL3. As the INSL3 gene has also been identified in pigs, and porcine LCs express INSL3 messenger RNA, the growth stimulatory effect observed by some investigators might have been related to INSL3 activity.[12] The other several studies have shown that IGF1 is required for proper development and functioning of the LCs in the developmental process of the testis.[5–7] The use of an organ culture with mouse gubernaculum for investigating hormonal control of undescended testis (UT) has failed, as no growth stimulatory effect could be induced by the addition of mouse testis. Furthermore, studies on in vitro skeletal muscle development showed that only a limited degree of differentiation can occur in vitro, probably because of the lack of innervation, growth factors, and extracellular matrix.[13,14]

The cremaster muscle (CM) is associated with the development of the gubernaculum,[15] and both are richly innervated by the genitofemoral nerve (GFN)[16]; division of the GFN inhibits the descent of the testis, and the gubernacular motor nucleus and the GFN are proposed to be sexually dimorphic through the effects of androgens.[17] The release of calcitonin gene-related peptide, which is present in excess amounts in males, through the GFN is proposed to guide the gubernaculum into the bottom of the scrotum.[18] An interruption in either of the phases has been believed to result in an UT. For example, the UT is more common among boys with neurogenic problems such as myelomeningocele.[19]

The major role of androgen, in addition to the factors discussed above, occurs in the second stage. The second stage of testicular descent, the inguinoscrotal phase, is arranged by a combination of hormonal and mechanical factors. During this stage, the enlarged gubernaculum anchors the testes near the inguinal canal. After that, androgens induce gubernacular regression by the disappearance of the extracellular matrix causing to a condensation of fibrous material and an increase in cell density.[20] The combination of regression of the gubernaculum and abdominal pressure caused by the growth of viscera has been claimed to push the testes down through the inguinal canal.[21]

Although the etiology of UT in human is unknown, it is hypothesized to be dependent upon a number of factors and most likely due to altered signaling of INSL3/relaxin/insulin-like family peptide receptor 2 (INSL3/RXFP2) and/or androgen receptor (AR) pathways, which are essential for gubernacular development.[22] INSL3/RXFP2 signaling is vital for testicular descent, while AR plays a more important role in genital development, but specific mutations of these genes are uncommon in UT.[23]

Androgens act indirectly on the gubernaculum[24] by masculinizing the sensory nucleus of the genitofemoral nerve. Calcitonin gene-related peptide (CGRP) released from the sensory branch of the nerve acts as a trophic agent for gubernacular development.[25,26] CGRP brings about rapid, rhythmic contractions of the rat gubernaculum in organ culture.[27] In an experimental rodent study, the distal end of the developing CM related to gubernaculum has been investigated regarding its growth. The bulb has been at the site of the most gubernacular growth, while mitosis in another part of the CM–gubernaculum complex has been more involved with possible differentiation of mesenchymal cells into muscle precursor cells and developing cells of the developing cremaster sac.[28]

Testicular development and probably its descent to the scrotum is a complicated process, which is why the INSR and the IGFR1 are required. LCs, under the control of IGF1, produces both androgens and INSL3, which are responsible for the masculinization of the urogenital system and testicular descent. We aimed to investigate whether IGFR1 exists in CM complex and is associated with normally descended testis as well as UT cases.

2. Materials and methods

Between July 2014 and June 2015, we obtained CM samples from the orchidopexy operation by dissection and mobilization of the spermatic cord (Undescended testis group; UTG), and from the orchiectomy operation for unilateral testicular torsion (Control group; CG). The study included 15 C M samples from 15 patients ranging in age from 1 to 6 years (mean, 1.6 ± 0.17) in the UTG, and 15 patients ranging in age from 9 to 12 years (mean, 11 ± 1.01) in the CG. The study was approved by the Institutional Ethics Review Board for Clinical Research of Bozok University (2015/44).

No hernias were detected in either group. Exclusion criteria included reported or previously known anomalies, including hypospadias or micropenis, or any defined syndrome in controls. The height and weight of the patients were in the normal range for age (65^{th}—75th percentile). The location of either testis was proximal, namely above the external inguinal ring (canalicular) in UTG, intraoperatively. Testis location in the CG was scrotal, and there was no underlying maldescent as a predisposing cause for acute torsion in all these cases.

Tissues were preserved in neutral formalin solution (10% dilution) after operation until the histopathological examination was performed. All samples were processed in an automated system (Excelsior ES; Thermo Scientific, Rockford, IL, USA), and paraffin blocks were prepared using the HistoStar embedding station (Thermo Scientific) at the Department of Pathology of the Bozok University School of Medicine. Two tissue sections 3—to 6-??m thick were obtained from each case using a microtome (Shandon-Finesse ME+; Thermo Scientific). One was stained with hematoxylin and eosin (H&E) using an automated slide staining machine (Varistain Gemini; Thermo Scientific), and the other was stained using an IGF1R antibody (1: 200 dilution, 1.0 ml concentrated mouse monoclonal, clone BC10, Biocare medical, Pike Lane Concord, CA, USA) in an automated immunohistochemical staining system (Leica-Bond Max, Leica Biosystems). The H&E— and IGFR1-stained slides were evaluated under a light microscope (BX53F; Olympus, Tokyo, Japan) by a pathologist. Muscles, nerves, and vessels in the operation materials were examined to determine whether they show IGFR1 expression immunohistochemically. Cytoplasmic and membranous staining were considered positive (Figs. 1—3). IGFR1 expression was scored as follows: score 0 = no staining; score 1 = mild staining; score 2 = moderate staining; score 3 = strong staining. All scores were analyzed using the t test for two groups with the same variance. A level of $p < 0.05$ was regarded as statistically significant. All statistical analyses were performed using SPSS ® for Windows ® (SPSS, Chicago, Illinois, USA).

3. Results

Necrotic or regenerating fibers or inflammatory cells were not encountered in any of the specimens. IGFR1 expression in muscle bundles of the CM was clearly present in the CG; however, there was nearly no IGF1R expression with brown staining in the majority of UTG samples (Fig. 1). The mean staining score (MSS) of IGFR1 in the CM was higher in the CG than in the UTG. This result was statistically significant ($p = 0.01$) (Table 1) (Fig. 4).

As in muscular tissue, the intensity of brown staining of IGFR1 expression in the nerves of CM was evident in most CG cases (Fig. 2). The MSS of IGFR in the nerve branches of CM was higher in the CG than the UTG, and this was also statistically significant ($p = 0.02$) (Table 1). IGFR1 expression in the vessel wall of the CM was relatively remarkable in the CG when compared with the UTG. Additionally, there was almost no brown-stained area as

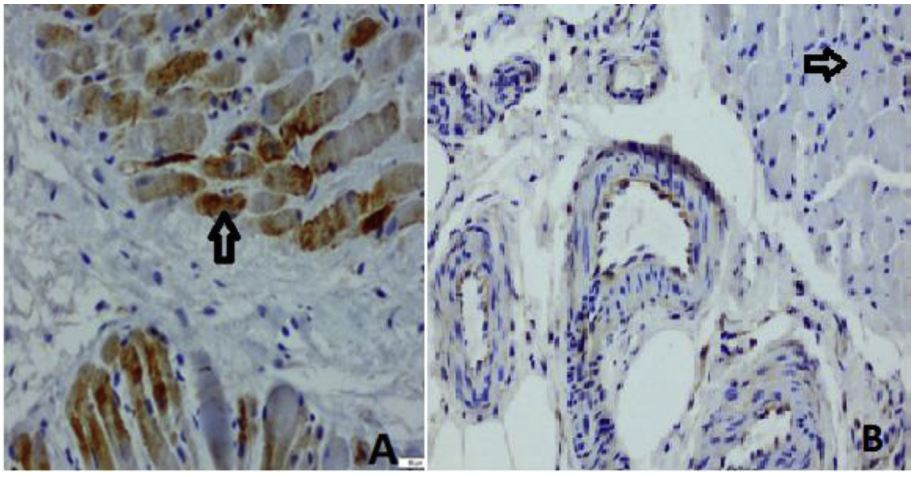

Figure 1 IGFR1 expression (brown staining) in muscle bundles of the CM (original magnification × 200). While brown staining (black arrow) of IGFR1 (**A**) was clearly present in the CG, there was almost no brown staining (original magnification × 200) in the UTG (**B**).

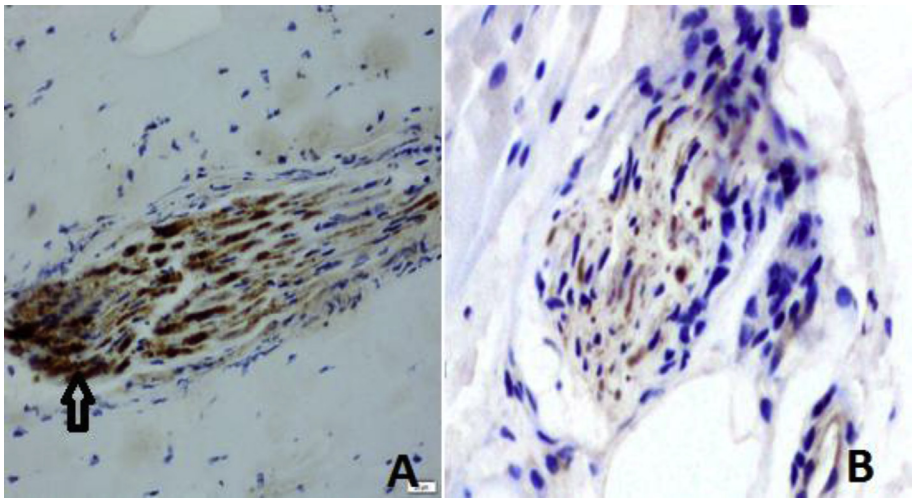

Figure 2 IGFR1 expression (brown staining) in nerve tissue of the CM (original magnification × 400). In the CG (**A**), brown staining of IGFR1 (black arrow) was more marked than in the UTG (original magnification × 400) (**B**).

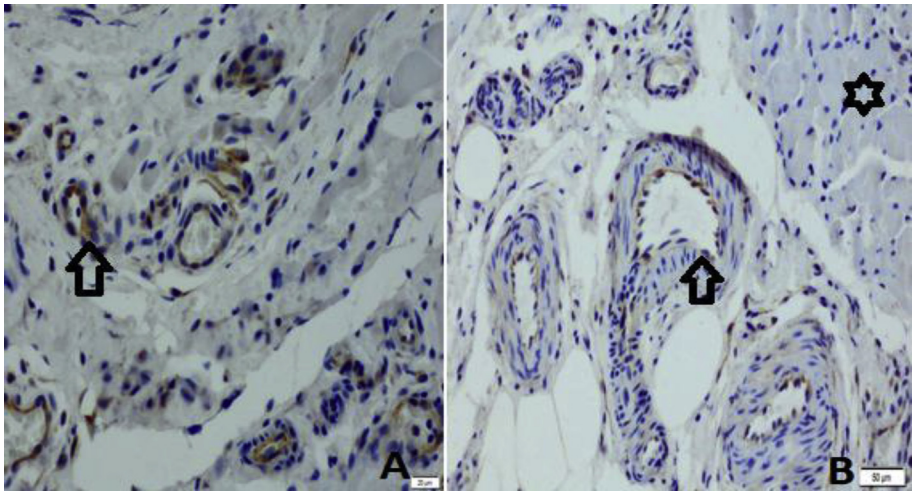

Figure 3 IGFR1 expression (brown staining) in the vascular tissue of CM (original magnification × 400). In the CG (**A**), brown staining (black arrow) was relatively more marked than in the UTG (original magnification × 400) (**B**). Additionally, almost no brown staining of IGFR1 (black asterisks) was seen in the muscle fiber of this section (**B**).

Table 1 Comparison of the amount of staining of the tissues with IGFR1 mean score.

	IGFR1 mean score of UTG group (n = 15); (mean ± S.D.)	IGFR1 mean score of CG (n = 15); (mean ± S.D.)	P Value
Muscle tissue	0.20 ± 0.41	1.06 ± 0.23	**0.01**
Vessel tissue	2.26 ± 0.88	2.46 ± 0.74	0.48
Nerve tissue	1.66 ± 1.23	2.60 ± 0.63	**0.02**

IGFR1 expression was also seen in the muscle fibers corresponding to the same area (Fig. 3). Although the MSS of IGFR1 was higher in the vessel walls of the CM in the CG than UTG, it was not statistically significant (p = 0.48) (Table 1).

4. Discussion

The insulin family of growth factors (insulin, insulin-like growth factors (IGF-1 and II), with small single-chain mitogenic polypeptides, provide necessary signals for the control of growth, metabolism, and reproductive functions. It also plays a significant role in the proper development and function of the testis. Although reproductive ability is controlled by the hypothalamic–pituitary–gonadal axis, the activity of local gonadal factors, such as those of the insulin/IGF family, regulate reproductive performance.[5,29,30] IGF-1 biosynthesis and action take place in the testis as well as in many organs and body systems. IGF-1 mRNA, protein, and specific IGF-1 receptors are present in the testis and have been detected in Leydig cells (LCs), peritubular cells, and spermatocytes.[31–36]

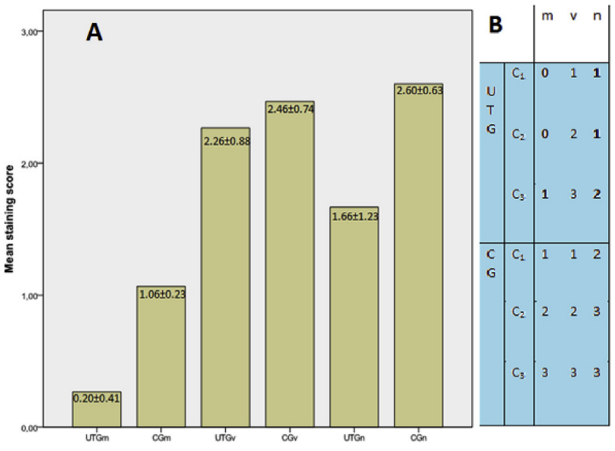

Figure 4 **A)** Distribution of mean staining score of IGFR1 in the Cremasteric muscle complex of UTG and CG (m: muscle; v: vessel; n: nerve tissue). **B)** IGFR1 staining score examples of 3 cases from UTG and CG ($C_{1,2,3}$: Case 1,2 and 3; score 0 = no staining; score 1 = mild staining; score 2 = moderate staining; score 3 = strong staining.).

The physiological effects of insulin, IGF1, and IGF2, including cell survival, proliferation, differentiation, and metabolism, are mediated through the activation of two related tyrosine kinase receptors: the insulin receptor (INSR) and the type-I insulin-like growth factor receptor (IGFR1). INSR and IGFR1 consist of heterotetrameric glycoproteins with two extracellular α subunits and two transmembrane β subunits linked together by disulfide bridges.[37,38] Insulin binds with its own receptors (INSR-A and INSR-B) with high affinity but also binds to IGFR1 and the hybrid receptors INSR-A/IGFR1 and INSR-B/IGFR1 with a lower affinity. Although the IGFR1 has different α and β subunits compared to the INSR, these subunits of $\alpha\beta$ dimers allow for hybrid receptors, which bind insulin, IGF1, and IGF2 with differing affinities.[38–40]

The insulin/IGF1 signaling system with two major pathways, ATK/PI3K and ERK/MAPK, is involved in several cellular processes, such as metabolism, cell growth, proliferation, and apoptosis. Activation of the INSR/IGFR1 system by insulin/IGF1 binding leads to autophosphorylation of the β subunits, and the receptor tyrosine kinase, consequently, phosphorylates IRS proteins on their tyrosine residues. This activation creates recognition areas for additional effector molecules containing SH2 domains, such as the p85 regulatory subunit of PI3K (which activates the AKT/PI3K pathway and is mainly responsible for the metabolic actions of insulin/IGF1) and GRB2 (which activates the ERK/MAPK pathway and primarily regulates cell growth and differentiation). As a result, these signals stimulate varied downstream biological effects, including mitogenesis, gene expression, glucose transport, and glycogen synthesis.[41–44]

In general, IGF1 plays a significant role in overall growth, whereas IGF2 is accepted to act during embryonic development.[5,29,30] However, it is not known whether this general pattern holds true for the testicular descent, development, and function. Further exploration is needed to determine the relative contributions of endocrine, paracrine, and autocrine effects of the IGFs in the testis.

IGFs are implicated in a variety of functions, including adrenogonadal development involving adrenal specification, testicular differentiation, and LC development and function.[1,2,4] The masculinization of the urogenital system and testicular descent depend on normal LCs producing INSL3 and androgen.[4] The gubernaculum, where the CM develops,[15] is richly innervated by the genitofemoral nerve (GFN).[16] Calcitonin gene-related peptide (CGRP) release from the sensory branch of the nerve acts as a trophic agent for gubernacular development.[25,26] As it has been shown that organ culture with mouse gubernaculum is required for investigating hormonal control of UT, a growth factor could be required for mouse testis.[13] For example, INSL3 and androgen are necessary for this process, but they both require an additional or a growth factor. In the current study, we investigated whether IGFR1 exists in CM complex. We detected IGFR1 in CMs of both the UTG and the CG. The density of IGFR1 was higher in the control group and the difference between groups was statistically significant ($p = 0.01$). Tanyel et al have examined the CM status in boys with UT. In these boys, atrophic angular fibers and atrophy have been detected in the CM of UT. Regarding altered or defective innervations of CM, the same study has shown that the defective innervations could cause the CM to act as an obstacle that contributes to the failure of testicular descent.[45] In the testis descent, androgens act indirectly on the gubernaculum[24] by masculinizing the sensory nucleus of the GFN. CGRP released from the sensory branch of the nerve serves as a trophic agent for gubernacular development.[25,26] CGRP causes rapid, rhythmic contractions of the rat gubernaculum in organ culture.[27] As Tanyel et al demonstrated, if there are the defective innervations in this cascade and the atrophic angular fibers of CM, both the CM and its nerve may have inadequate development, exposing the failed growth stimulation. Therefore, in addition to the effect of CGRP and androgen, IGFR1 on the CM and nerve branch, which we have identified in the present study, may also be effective in this cascade as a pivotal growth receptor that is responsive to growth factors. As substantial evidence of this, UT is frequently encountered among male children with neurogenic problems, including myelomeningocele.[19]

Gubernacular development is related to CM, and its rhythmic contractions occur through the release of CGRP from the sensory branch of the GFN, which contributes to the testicular descent.[27] When the nerves of CM specimens were examined, IGFR1 was detected in our study. The density of IGFR1 was higher in the CG than that in the UTG, and it was statically significant ($p = 0.02$). GFN may be effective on CM and gubernaculum via CGRP. However, in the developmental stages of these nerves, a developmental defect could limit its effects and cause an aberration in the development. That is, both of GFN and gubernaculum-CM complex are required the growth stimulation to develop properly. Unless GFN develops enough, the gubernaculum-CM complex is not induced and don't develop due to presumably a failed innervation by the GFN. The use of an organ culture with mouse gubernaculum for investigating hormonal control of UT has failed, as no growth stimulatory effect could be induced by the addition of mouse testis. On

the meanwhile, in the same study, the muscular layer of the gubernaculum in vitro, even after coculture with a testis, has been detected thinner compared with the in vivo situation. This result was also found in studies on in vitro skeletal muscle development.[13,14] According to the above information, if the insulin, IGF1, and IGF2 are able to accept as the effective stimulants, IGF1R with the hetero-tetrameric structure[38–40] seems to be the best candidate as the receptor.

A cell culture study has shown that IGF-1 promotes angiogenesis by stabilizing neovessels.[46] Our study demonstrated that the vascular wall of the specimens of the UTG and CG had IGF1R. However, this was not statistically significant ($p = 0.48$) regarding the mean density score. To the best of our knowledge, previous studies have made no determination on whether a vascular development defect is present in the CM complex or not. In our study, although the IGFR1 expression in the vessel wall of the CM was relatively remarkable in CG when compared with UTG, there was no significant difference concerning IGFR1 expression between the UTG and CG. That is, no aberration was detected in the vascular development of the CM complex in either group.

Recent studies in various tissues have shown that INSR and IGF1R are able to translocate to the cell nucleus and function like transcription factors by binding to specific promoters and enhancers to control gene expression.[46] The IGFs plays a crucial role not only in mediating the proliferation of precursor LCs and the establishment of a normal number of adult LCs, but also in the androgenic capacity of adult LCs.[5,6] Fetal LC development is one of the key points in the cascade whereby the above action occurs. Fetal LCs produce both androgens and INSL3, which are responsible for the masculinization of the urogenital system and testicular descent.[4]

Experiments in LH-receptor-knockout mice show that androgen treatment increases INSL3 receptor expression in the gubernaculum—CM in an androgen-receptor-dependent fashion. The presence of both INSL3 and testosterone is required in the reorganization of the gubernaculum, and an addition of an INSL3 receptor antagonist halts androgen-induced inguinoscrotal testicular descent. Additionally, testosterone has been shown to stimulate INSL3 expression in a human LC line.[47,48] Taken together, these suggestions have shown that during transabdominal and inguinoscrotal testicular descent, the INSL3—testosterone-receptor interaction is closer than previously supposed. The significance of this interplay in humans, however, has usually been shown in vivo experimental models. In the current study, we demonstrated the presence of IGFR1 in human CM. Furthermore, the density of IGFR1 in the CM of UT patients was lower than that of the CG. We have supposed that IGF1R both affects this process and is affected by this cross-linked relationship.

The relationship between androgen and IGF1 receptors has mainly been investigated in prostate cancer. An intracrine-positive feedback between IGF1 and androgen receptor (AR) signaling has been involved in prostate cancer cells. Liganded AR upregulates IGF1 receptor expression causing higher IGF1 signaling in prostate cancer cells.[49] Tanyel et al have suggested that the expression of AR is increased in CM associated with UT. As AR is regulated by androgens in a tissue- and cell type—specific fashion, the increase might support the decrease in androgen effects. Androgen receptor can be activated ligand-dependently by androgens and ligand-independently by other hormones and various growth factors.[50] Taken together, AR and the probable growth factor, as well as its receptor, may interact in the process of testes descent.

5. Conclusions

Defective androgen action can cause cryptorchidism, but the etiology currently remains unknown.[51] INSL 3, CGRP, androgen, and its receptor contribute the testis descent. As an affected and promoting structure, CM with gubernaculum requires growth and multiple stimulators for the testis descent process. IGFR1, which has a significant role from adrenogenital development to the testis maturation, influences this cascade during testis development and descent. IGFR1 with heterotetrameric receptor via IGF1, IGF2, insulin, and probably androgens contribute to the remodeling and development of CM as well as the testis descent. There is the need for further investigations whether IGFR1 and other etiological factors are the relations to each other. Moreover, there is the requirement for the quantitative measurement of the density of IGFR1 and also other substances such as INSR, INSL3, and PI3K pathway substrates using mRNA hybridization methods in future research.

Funding

This work was supported by the Bozok University Institution of Scientific Research Projects [Grant numbers, 2016TF/A221].

Conflicts of interest

None.

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