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Molecular signals governing cremaster muscle development: Clues for cryptorchidism

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ABSTRACT

Background/Aim: Cryptorchidism affects 2-4% of newborn boys. Testicular descent requires the gubernaculum to differentiate into cremaster muscle (CM) during androgen-mediated inguino-scrotal descent, but the cellular mechanisms regulating this remodeling remain elusive. β-Catenin, a marker of canonical Wnt signaling, promotes myogenic genes and cellular adhesion. We aimed to determine if androgen receptor (AR) blockade altered β-catenin and its downstream myogenic proteins within the CM.
Method: Gubernacula from male rats (n = 12) and rats treated with anti-androgen, flutamide (n = 12) at E19, D0, D2 were processed for immunohistochemistry. Antibodies against β-catenin, embryonic myosin, and myogenin were visualized by confocal microscopy.
Results: At E19, β-catenin immuno-reactivity (IR) localized to the CM membrane. By D2, cytoplasmic β-catenin-IR was noted with overall β-catenin-IR decreasing. Myogenic proteins resided primarily in cells containing β-catenin on their plasma membrane. Embryonic myosin-IR was high at E19 and then decreased by D2, while myogenin-IR increased. AR blockade increased cytoplasmic β-catenin at D2 and reduced levels of both myogenic proteins.
Conclusion: Myogenic proteins are present in CM cells containing β-catenin. AR blockade did not alter cellular adhesion via β-catenin. In contrast, blocking AR prevented β-catenin entering the nucleus and impaired CM myogenesis. Mutations in this pathway may result in idiopathic cryptorchidism.

Cryptorchidism is one of the most common genital anomalies that affect 2–4% of newborn males [1]. Testicular descent into the scrotal position relies on a ligament (the gubernaculum) that is composed of primitive mesenchymal cells. These cells respond to hormonal cues that cause proliferation and differentiation into striated muscle that establishes the cremaster muscle (CM). Androgen is one of the primary hormones governing CM development and regulates the remodeling of the gubernaculum during CM formation; however, the exact molecular mechanisms controlling mesenchymal cell differentiation into the CM remain unknown.

During the second, or inguino-scrotal phase of testicular descent, the rodent gubernaculum remodels in response to androgen causing the repositioning of the loose mesenchyme [2]. During this phase, the mesenchymal cells undergo differentiation and proliferation that results in the formation of striated muscle that constitutes the CM. The gubernaculum then everts into the inguinal subcutaneous tissue and migrates towards the scrotum, pulling the testes along with it. Recent research using microarray analysis has suggested that Wnt signaling working in conjunction with androgen receptor (AR) may contribute to CM formation and testicular descent, as a defect in the Wnt signaling pathway and AR both produce intra-abdominal testis, which is a phenotype commonly associated with patients presenting with complete androgen insensitivity syndrome [3,4].

Androgens are thought to act in a Wnt-dependent manner by stabilizing the key effector β-catenin [5,6]. During striated muscle development, β-catenin is known to participate in 2 molecular roles: gene transcription and cell-cell adhesion [7]. Gene transcription occurs in the nucleus. When a Wnt ligand binds to a canonical Frizzled receptor β-catenin stabilizes within the cytoplasm where it then translocates through the nuclear pore, bound to be liganded AR. Once in the nucleus it then interacts with T-cell factor and lymphoid enhancing factor (TCF/LEF) to facilitate the transcription of Wnt-responsive genes. The Wnt-responsive genes transcribed through this pathway have been implicated in all facets of cellular homeostasis that include cellular proliferation, differentiation as well as initiating events that contribute to myogenesis [8]. Secondly, β-catenin is also

Abbreviations: IR, immunoreactivity; CM, cremaster muscle; E, embryonic day; D, postnatal day; AR, androgen receptor; TCF, T-cell factor; LEF, Lymphoid enhancing factor; IGF-1, insulin like growth factor-1.

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Table 1
Primary and secondary antibodies.

	Company/catalogue number	Raised in/ clonality	Working conc. (vol/vol)
Primary antibody			
Anti-β-catenin	Abcam, ab2365	Rabbit/polyclonal	1/200
Myogenin	Abcam, ab1835	Mouse/monoclonal	1/200
Embryonic myosin	DSHB, BF-45	Mouse/monoclonal	1/200
Secondary antibody			
Alex 594	Invitrogen A-11001	Donkey/mouse	1/400
Alex 488	Invitrogen A-11008	Donkey/rabbit	1/400
DAPI	Sigma-Aldrich, D9564	N/A	1/1000

known to regulate cellular adhesion during myogenesis by regulating boundary formation between cells. This achieved by the membrane-bound protein, cadherin [9]. The cytoplasmic portion of the cadherin protein has β-catenin binding sites allowing β-catenin and cadherin proteins to form active cadherin-catenin complexes. This complex binds in the plasma membrane of cells in conjunction with α-catenin, in turn allowing neighboring cells to fuse together in a coordinated fashion. This process enables multilayered muscle spindles to form, facilitating myogenesis [10].

During the formation of the CM, conditional knock down of β-catenin caused a failure in CM myogenesis resulting in intra-abdominal cryptorchidism [11]; however, how β-catenin regulates mesenchymal cell differentiation into the CM is not completely known. As previous authors have implicated β-catenin as the major protein regulating CM formation, we aimed to determine the precise localization of β-catenin during inguino-scrotal descent and decipher its role in relation to its spatiotemporal location and assess the effect of AR blockade on its downstream myogenic targets.

1. Methods

Sprague-Dawley rats were purchased from a commercial supplier and housed in the institute's Animal Research Laboratory in standard shoebox cages. Animals were maintained in a temperature-controlled atmosphere with a 12-hour light-dark cycle and fed commercial rat chow and water *ad libitum*. Ethical approval was gained from the Institutional Ethics Committee (license number A644) and with correct care taken in accordance with the National Health and Medical Research Council animal ethics guidelines.

Male fetuses were collected at embryonic day 19 (E19; n = 4) (E0 = day vaginal plug found). One group of dams were time-mated and allowed to deliver where male offspring were collected at birth (D0; n = 4) and D2 (n = 4). A separate group of dams (n = 3) received the anti-androgen, flutamide (75 mg/kg bodyweight) on gestational days E16 to E19 inclusive (12 male pups from flutamide group collected in total at the same time points mentioned above). Animals were sacrificed by Lethabarb injection (2 mL/kg; Vertak pk, Australia) after being anesthetised with 5% isofluorane. Pups were removed by hysterectomy, placed on ice for 10–15 minutes and bisected at the level of the umbilicus with the lower body fixed in 4% paraformaldehyde for 48 hours at 4 °C. Specimens were paraffin processed, cut, and prepared for immunohistochemistry according to a previously described protocol [12]. Single- and double labelling of gubernacular sections occurred with the antibodies described in Table 1. Slides were mounted using Mowiol and stored in the dark at 4 °C. A Leica LSM-2 confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to obtain fluorescent images.

Images were assessed using ImageJ (ImageJ version 1.43u, Wayne Rasband, National Institute of Health, USA). The fluorescence values for each protein in the CM were calculated for images taken at ×63 magnification. Values were tabulated as the ratio against 4′,6-diamidino-2-phenylindole (DAPI) that labels cell nuclei and allows the fluorescence intensity to be related to the number of cells within each image. Ratios were initially calculated in Microsoft Excel and then transferred into SPSS (V.21) for statistical analysis. Data were compared using the Mann-Whitney U test with significance was assigned at $P < 0.05$. Data were then expressed as median and inter-quartile range (IQR) (Table 2 for all data sets).

2. Results

2.1. β-Catenin localises to the developing CM on the cell surface

Labelling experiments showed that β-catenin immuno-reactivity (IR) was most prevalent in the CM with β-catenin-IR residing on the whole plasma membrane of CM cells in E19 ($n = 4$) and D0 specimens ($n = 4$). β-catenin-IR appeared cytoplasmic in D2 specimens that labelled ubiquitously throughout the entire CM ($n = 4$). Additionally, β-catenin-IR appeared to fragment by D2, a time when the gubernaculum is everting before migration into the scrotum (Fig. 1). The overall IR pattern in the time-points investigated suggests that β-catenin is highest at E19, but declines by D2 ($n = 12$, Table 2, Fig. 2).

2.2. β-Catenin-positive CM cells express the embryonic myosin protein

In E19 specimens, β-catenin-IR and embryonic myosin-IR co-localise at the cell-cell contact sites with this diminishing by D2. Embryonic myosin labeled only within the cytoplasm of differentiating CM cells containing β-catenin on their cell surface. Cells that lacked β-catenin on the plasma membrane showed no embryonic myosin-IR (Fig. 3).

2.3. Androgen blockade causes increased intra-cytoplasmic β-catenin IR at D2

The localization of β-catenin-IR at the plasma membrane at E19 did not change when AR was blocked. However, in D2 specimens, a significant increase in β-catenin-IR was observed in the cytoplasm of CM cells when AR was inhibited ($n = 4$ per group, Table 2, Fig. 4A).

Table 2
Comparative statistical analysis of fluorescence intensity in each gubernacula group.

		n	SD	IQR	M	Mann-Whitney *U*	*P*
Fig. 2	E19 Control & D2 Control	8	0.0919	0.1687	0.1725	1	<.05
Fig. 4A	E19 Control & E19 Flutamide	8	0.0977	0.1426	0.3049	7	.773
	D2 Control & D2 Flutamide	8	0.144	0.2307	0.2005	0	<.05
Fig. 4B	E19 Control & E19 Flutamide	8	0.2646	0.4996	0.438	0	<.05
	D2 Control & D2 Flutamide	8	0.1552	0.2282	0.0361	1	<.05
Fig. 4C	E19 Control & E19 Flutamide	8	0.0432	0.0476	0.0114	0	<.05
	D2 Control & D2 Flutamide	8	0.0509	0.0863	0.0274	0	<.05

*SD = standard deviation, IQR = interquartile range, M = median.