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.

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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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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 is highest at E19, but declines by D2 [n=4 per group, Table 2, Fig. 4A].

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 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 1
Primary and secondary antibodies.

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<th>Company/catalogue number</th>
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<th>Working conc.</th>
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Table 2
Comparative statistical analysis of fluorescence intensity in each gubernacula group.

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</table>

*SD = standard deviation, IQR = interquartile range, M = median.
2.4. Androgen blockade inhibits embryonic myosin IR

Embryonic myosin-IR was reduced in all time-points in AR blocked specimens when compared to controls. By D2, embryonic myosin-IR was severely decreased and markedly less than in control animals \( (n = 4 \text{ per group, Table 2, Fig. 4B}) \).

2.5. Myogenin IR declines in flutamide-treated CM

Myogenin-IR increased in the nuclei by D2 in control animals. However, when AR was blocked there was little myogenin-IR across all ages. By D2, a stage where myogenin-IR was seen to increase in controls, there was significantly less myogenin-IR in flutamide-treated CM \( (n = 4 \text{ per group, Table 2, Fig. 4C}) \).

3. Discussion

Emerging evidence suggests that the key effector of the canonical Wnt pathway, \( \beta \)-catenin, is essential for testicular descent [13] and that it interacts with AR to enable expression of downstream myogenic proteins. \( \beta \)-Catenin is highly conserved throughout evolution, and the intracellular cascade that regulates its translocation to either the nucleus or the plasma membrane hinges on the molecular machinery that stabilizes it in the cytoplasm. Ligands such as Wnt1, Wnt3a and Wnt5a, initiate the stabilization of \( \beta \)-catenin [14]. This stabilized \( \beta \)-catenin then functions in both myogenic gene transcription as well as cellular adhesion; enabling cells to adhere to one another in a coordinated fashion ensuring muscle fiber maturation.

This novel study clearly shows that \( \beta \)-catenin localizes to the plasma membrane of CM cells at E19 then membranous \( \beta \)-catenin decreases and fragments when the gubernaculum begins to evert and migrate towards the scrotal position. This localization was independent of androgen. However, blocking AR resulted in changes in \( \beta \)-catenin levels in the cytoplasm of CM cells by D2. This occurred at the same time the myogenic proteins embryonic myosin and myogenin were reduced. Thereby showing that CM maturation is inhibited concurrently with \( \beta \)-catenin accumulation, providing an insight into the molecular mechanisms contributing to CM mesenchymal cell differentiation.

In this study, \( \beta \)-catenin labelling was carried out at E19, D0 and D2, being the start of the inguino-scrotal stage of descent, to determine \( \beta \)-catenin's spatial-temporal arrangement within the gubernaculum. \( \beta \)-catenin-IR was present in the developing CM but not the undifferentiated mesenchymal cells of the gubernacular core. We observed that \( \beta \)-catenin localized to the plasma membrane

![Fig. 1. \( \beta \)-Catenin in control CM. (A) Control CM at E19 showing \( \beta \)-catenin localising to the plasma membrane (arrow). (B) D2 CM cell displaying \( \beta \)-catenin-IR beginning to fragment at the same time the gubernaculum begins to evert into the scrotum (arrow). Scale bar 10 \( \mu \)m.](image1)

![Fig. 2. Mean \( \beta \)-catenin-IR in CM control specimens. \( \beta \)-Catenin-IR is highest at E19 with a significant decline by D2 \( (p < 0.05) \).](image2)

![Fig. 3. Embryonic myosin in control CM. At E19 in control specimens, \( \beta \)-catenin-IR (green) and embryonic myosin-IR (red) in CM show a possible correlation. When \( \beta \)-catenin is present between cell-cell junctions both neighboring cells contain colocalized myosin-IR (yellow arrow). Whereas, when \( \beta \)-catenin is lacking, this coordinated immuno-reactivity pattern is absent between neighbouring cells (white arrow).](image3)
of CM cells residing between the cell-cell contact sites, most likely to be the cellular junction abundant in adhesive proteins, the adherens junctions.

Previous reports have suggested that $\beta$-catenin localization to the plasma membrane indicates active adherens junctions. Finnermann et al. observed that once gene transcription had ceased in proliferating premyogenic cells, $\beta$-catenin localized to the adherens junctions [15]. They hypothesized that cadherin proteins and $\alpha$-catenin form a dimer and sequester cytoplasmic $\beta$-catenin and link it to the actin cytoskeleton where it then enables cellular coordination. At E19, localization of $\beta$-catenin on the plasma membrane is consistent with its role in cellular adhesion and suggests active adherens junctions have formed during CM myogenesis. Moreover, decreased $\beta$-catenin-IR by D2 on the cell membranes, accompanied by a fragmenting appearance of $\beta$-catenin fluorescence suggests that adhesion between CM cells is breaking down. We hypothesize that active adherens junctions are separating to allow gubernacular eversion and migration to the scrotum, as this loss in adhesion occurs at the correct time to enable the gubernaculum to evert.

Additionally, double-labelling experiments showed that $\beta$-catenin co-localized with the primary muscle cell marker, myosin. At E19, $\beta$-catenin localization to the plasma membrane appears to dictate the expression of embryonic myosin, with CM cells that lacked $\beta$-catenin on the cell surface lacking the embryonic myosin protein within their cytoplasm. It is tempting to speculate that committed cells expressing the myosin protein influence the pathway of differentiation of their uncommitted neighbors, aided by $\beta$-catenin at the adherens junctions. It is hypothesized that once cells express the correct maturation markers, muscle cells would then communicate with their microenvironment and adhere to one another to form the multinucleated, maturing CM fibers. This appears to be independent of AR as when AR was blocked, $\beta$-catenin on the cell surface was unaffected. Taken together, this suggests that by sending intercellular signals through adhesive receptors, such as $\beta$-catenin, CM cells are able to elicit the spatially coordinating events that are required for coordinated myogenesis.

Furthermore, the increase in cytoplasmic $\beta$-catenin in the CM observed in D2 control specimens is consistent with its role as a myogenic transcript promoter and suggests that in D2 CM, $\beta$-catenin is beginning to accumulate to initiate the transcription of myogenic proteins to enable CM maturation. However, the generation of myogenic transcripts relies on $\beta$-catenin reaching the nucleus to form a complex with TCF/LEF. This nuclear translocation of $\beta$-catenin is hypothesized to be dependent on interactions with AR, as binding to AR allows $\beta$-catenin to travel through the nuclear pore [16]. When flutamide specifically blocked AR before the start of inguino-scrotal descent, $\beta$-catenin-IR increased in the cytoplasm of the CM cells at D2 when compared to controls. This suggests that $\beta$-catenin is not able to bind to AR, reducing nuclear translocation, causing it to remain in the cytoplasm. This increased cytoplasmic accumulation could lead to impaired transcription of myogenic genes within the CM.

In support of this, after flutamide treatment, embryonic myosin- and myogenin-IR were severely reduced; suggesting primary myofiber formation and differentiation of mesenchymal cells was delayed. This delay coincides with altered $\beta$-catenin levels at D2. We therefore hypothesize that embryonic myosin and myogenin are reliant on signals provided by $\beta$-catenin, AR and TCF/LEF in the nuclei of CM cells to initiate myogenic transcription and when this process is interrupted, CM maturation is inhibited.
The hypothesized interactions between β-catenin and AR remains a topic of debate with some reports suggesting that insulin-like growth factor-1 (IGF-1) plays a part in β-catenin nuclear translocation [17]. Verras et al. aimed to determine which molecules were implicated in enhancing β-catenin translocation in prostate cancer cells in culture by manipulating culture conditions to observe the effects of IGF-1. They observed that β-catenin still translocates to the nucleus bound to IGF-1. However, the difference observed between the experiments completed by Verras et al., and the current study may be due to the manipulated culture conditions, as well as the specific cell line used and hence, extrapolation to the CM may not be warranted and may require further experimentation to apply to CM myogenesis.

This study shows the canonical Wnt pathway is active in the gubernaculum during inguino-scrotal descent, as indicated by the presence of β-catenin in the CM. Blocking AR leads to β-catenin accumulating in the cytoplasm and reduced myogenic proteins suggesting androgen controls translocation of β-catenin to the nucleus and myogenic gene transcription before evisceration and migration towards the scrotum. β-catenin is also involved in CM cellular adhesion, enabling cremaster cells to adhere to one another during gubernacular remodeling. These results suggest canonical Wnt proteins interact with androgens during inguino-scrotal descent, providing clues to the complex etiology of congenital cryptorchidism.

References


Discussion

Discussant: Paolo De Coppi (Great Ormond Street Hospital, London): Have you looked at controlling the muscle with an anti-androgen as a factor (or an effect?) in other skeletal muscles and secondly, using muscle when there is no differentiation there is proliferation – did you look at proliferation of these cells?

Response: Mr. Szarek: We did not do a control muscle because the cremaster muscle is different from other skeletal muscles in the body and matures quite late, and differs in the firing frequency in its contractions. I wasn’t going to use the anterior abdominal wall as a control because I don’t think that would be a good solid control. A previous study from our lab has used Ki67 to mark proliferation. The gubernaculum does have a proliferation zone, which is the progress zone, but because I looked at the cremaster, this is where the cells should be differentiated and there shouldn’t be proliferation.

Discussant: Clare Rees (Royal London Hospital): How do any of the genetic pathways explain unilateral cryptorchidism which is the most common form that we see?

Response: Mr. Szarek: There are two gubernacula, one on the left and one on the right, so there is a balance and when there is a problem on the key signalling pathway, this has gone wrong.