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Mathematical modelling of gubernaculum during inguino-scrotal migration shows limb bud characteristics

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Abstract

Purpose: The gubernaculum is postulated to grow like an embryonic limb bud during inguinoscrotal descent in rodents. Recently, modelling of limb bud growth suggests the undifferentiated, distal "progress zone" provides molecular morphogenic signals, rather than cell division, as previously thought. We aimed to develop a mathematical gubernacular growth model, hypothesising that it would mimic limb buds through evolutionary conservation.

Methods: Histology was done on Sprague–Dawley rats (day 2, 8; n = 7/group) to determine gubernacular length, width, cell density in distal growth centre, middle and proximal cremaster muscle. Analysis of measurements enabled gubernacular growth modelling under variable growth centre sizes/densities, assuming no apoptosis.

Results: Modelling found that gubernacular growth occurred mostly within cremaster muscle, rather than primarily in the undifferentiated mesenchymal tip, despite its higher mitotic rate. The growth centre accounted for ≤ 10% of total gubernacular enlargement/elongation.

Conclusions: These results suggest the gubernaculum elongates by proliferation throughout cremaster muscle like a limb bud. The distal undifferentiated tip may provide signalling molecules for growth, which could be a fruitful source for causes of failed migration/elongation in cryptorchidism.

Descent of the testis is a complex process. Our current theories suggest that there are two main phases of testicular descent, both involving the caudal genito-inguinal ligament, known as the gubernaculum. In the first, or transabdominal phase, insulin-like hormone 3 (Insl3) from the Leydig cells controls proliferation of the mesenchyme in the gubernaculum (the so-called "swelling reaction") which holds the testis near the groin as the fetus grows between 8 and 15 weeks. In the second, or inguinoscrotal phase, the gubernaculum protrudes from the abdominal wall at about 25 weeks' gestation, and then elongates until it reaches the scrotum. The cremaster muscle develops inside the gubernaculum. This complex morphological change is mediated by androgens which act partly indirectly via the genitofemoral nerve releasing a neuropeptide, Calcitonin gene-related peptide (CGRP) [1].

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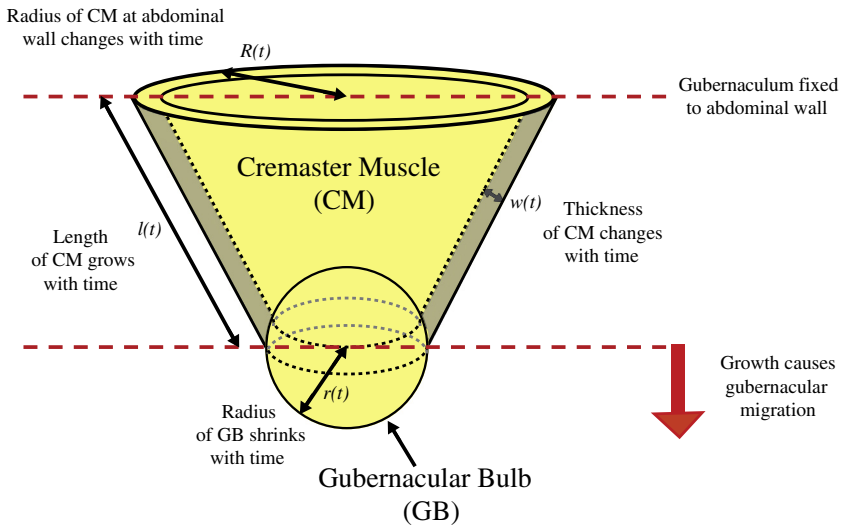

Fig. 1 Schematic diagram of the growth of the gubernaculum. Apical growth occurs at the distal end due to proliferation within the gubernacular core, or bulb. It was assumed that the cremaster muscle (CM) grows by 2 mechanisms: (1) all CM cells undergo mitosis at the same rate, and (2) all the gubernacular core or bulb (GB) cells undergo mitosis at the same rate, and some of these cells are exported into the muscle (CM), where they adopt the mitotic rate within the muscle.

During the second phase in rodents the gubernaculum is hollowed out by the developing processus vaginalis, but it remains connected to the testis by a central cord within the processus vaginalis. The cremaster muscle develops in the outer (annular) wall of the gubernaculum, forming a "cremaster sac", while the distal end of the gubernaculum has a solid mesenchymal tip. To reach the scrotum, the gubernaculum needs to grow in size and length with significant cellular proliferation. During outgrowth the gubernaculum has many characteristics of an embryonic limb bud, including the undifferentiated mesenchymal tip, which has some qualities similar to the "progress zone" of the limb bud [2-4].

Recent studies suggest that the tip or "bulb" of the gubernaculum contains a significantly higher percentage of active, dividing cells than any other region of the gubernaculum, hence the proposal that the bulb is its growing end [5]. This project aimed to determine how the gubernaculum grows by mathematical modelling, to predict the contribution of the undifferentiated gubernacular bulb to overall growth of the cremaster muscle during the inguinoscrotal phase of descent.

1. Methods

Sprague–Dawley rats were housed in conventional microisolator cages and fed tap water rat cubes (UV-sterilised, Barastoc, Pakenham, Victoria) ad libitum. Male offspring (n = 7) were collected at days 2 and 8 (day of birth is defined as D0) and prepared for histological examination by fixation of the pelvis in 4% paraformaldehyde in phosphate-buffered saline. Five micron sagittal sections of the inguinoscrotal region and gubernaculum were prepared and stained with H&E, as described previously [6].

In each gubernaculum, the cell density (cell number/mm^3) was determined in (1) the proximal cremaster muscle (CM) near the abdominal wall; (2) the distal (CM), near the end of the processus vaginalis; (3) the undifferentiated gubernacular tip or bulb (GB). In addition, we measured the radius of the cremaster sac at the inguinal ring proximally and the distance from the abdominal wall to the tip of the gubernaculum, to obtain an estimate of length at the two times.

1.1. Mathematical model and analysis

To model the growth of the neonatal rat gubernaculum, we considered the gubernaculum to consist of a cone-shaped "cremaster sac" made up of cremaster muscle cells attached

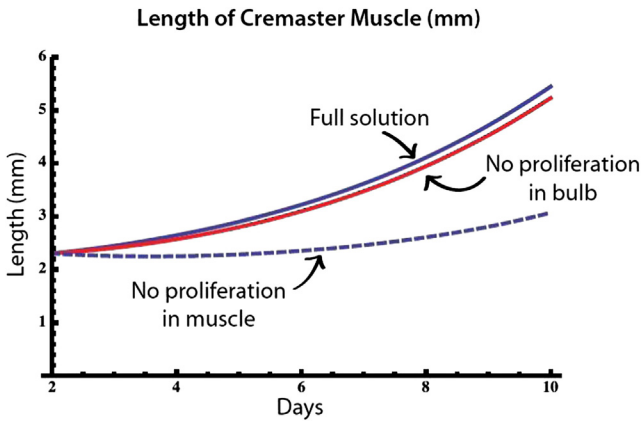

Fig. 2 Length of the cremaster muscle (mm) as a function of time (days). Solid (blue) line: full solution. Dashed (blue) line: length of the cremaster muscle when proliferation of the cremaster muscle is sutured off. Solid (red) line: length of the cremaster muscle when proliferation in the gubernacular bulb is switch off.

to the abdominal wall at the proximal end, and a sphere-shaped gubernacular bulb at the distal end (Fig. 1). The position of the abdominal wall remained fixed.

To consider the increase in the number of cells in the cremaster muscle (CM), it was assumed that the cremaster sac grows due to two mechanisms: (i) all the cremaster muscle (CM) cells undergo mitosis at the same rate and (ii) all the gubernacular bulb (GB) cells undergo mitosis at the same rate and some of these cells are exported into the CM [7]. When these cells enter the CM, they adopt the mitotic rate within the muscle.

$$\frac{d}{dt}\,(\text{Number of cells in CM}) = (\text{Mitotic rate in CM}) \times (\text{Number of cells in CM}) + (\text{Mitotic rate in GB}) \times (\text{Mitotic rate in GB}).$$

This equation can be written as

$$\frac{d}{dt}[V_c(t)\rho_c(t)] = m_c(t)V_c(t)\rho_c(t) + m_b(t)V_b(t)\rho_b(t), \quad (1)$$

where $V_c(t)$ and $V_b(t)$ are the volume of CM and GB respectively, $\rho_c(t)$ and $\rho_b(t)$ are the density of CM and GB respectively, and $m_c(t)$ and $m_b(t)$ are the mitotic index of the cells in the CM and GB respectively. Rearranging we obtained an expression for the rate of change of CM volume:

$$\frac{dV_c(t)}{dt} = V_c(t)\left(m_c(t) - \frac{1}{\rho_c(t)}\frac{d\rho_c(t)}{dt}\right) + \frac{\rho_b(t)}{\rho_c(t)}m_b(t)V_b(t). \quad (2)$$

The CM and GB volumes are rewritten in terms of the length $l(t)$ of the CM, width $w(t)$ of the CM, the radius of the cremaster sac at the abdominal wall $R(t)$ and the radius $r(t)$ of the GB bulb:

$$V_c(t) = \pi w(t)[R(t) + r(t) - w(t)]\sqrt{l(t)^2 - (R(t) - r(t))^2}, \quad (3)$$

$$V_b(t) = \frac{4\pi r(t)^3}{3}. \quad (4)$$

Substituting Eqs. (3)–(4) into Eq. (2) we obtain an ordinary differential equation for the rate of change of length of the CM:

$$\frac{dl}{dt} = \frac{(R-r)(\dot{R}-\dot{r})}{l} + \frac{4r^3 m_b \rho_b \sqrt{l^2-(R-r)^2}}{3wl\rho_c(R+r-w)} + \left[\frac{l^2-(R-r)^2}{l}\right]\left[m_c - \frac{\dot{\rho}_c}{\rho_c} - \frac{\dot{w}}{w} - \left(\frac{\dot{R}+\dot{r}-\dot{w}}{R+r-w}\right)\right], \quad (5)$$

where $\dot{r}$ represents the time derivative of the function r etc. Since the radius of the gubernacular bulb decreases with time, cells are also being pushed out of the gubernacular bulb and into the cremaster muscle as a result of this shrinkage to maintain the correct cell density.

From experimental data and previously reported values [5,8] (Tables 1–3), expressions for $m_b(t)$, $m_c(t)$, $\rho_c(t)$, $\rho_c(t)$, $w(t)$, $r(t)$ and $R(t)$ were fitted. Therefore to determine the length of the CM, we solve for $l(t)$ in Eq. (5) with an appropriate initial condition [$l(2) = 2.4 - 0.28\sqrt{2}$ mm].

Turning off cell proliferation within the gubernacular bulb: We set $m_b(t) = 0$ in Eq. (5) to determine the growth of the cremaster muscle if there was no contribution to growth from the gubernacular bulb.

Turning off cell proliferation within the cremaster muscle: We set $m_c(t) = 0$ in Eq. (5) to determine the growth of the cremaster muscle if there was no contribution to growth from the cremaster muscle i.e. no mitosis in the CM.

From the experimental data, expressions for the mitotic rates, the density and geometric properties of the tissue were determined using regression techniques. The differential equation for the length $l(t)$ was solved with appropriate initial length, using Mathematica.

To test the effective contribution of the GB and CM components, we switched off the cell proliferation in one of these components and compared it with the solution when the two components contribute to the elongation of the CM.

2. Results

The cell density (cells/mm^3) was determined from cross-sections of the undifferentiated gubernacular bulb, proximal cremaster and distal cremaster at days 2 and 8, which span the inguinoscrotal migration phase of the rat gubernaculum. The results are shown in Table 1. The size of the gubernaculum (length, cremaster width, gubernacular bulb area and radius) was determined from sagittal sections at days 2 and 8 (Table 2).

The mitotic rates in the gubernacular bulb and proximal cremaster muscle were derived from Tables 1 and 2 in Ng et al. 2005 [8], and are shown in Table 3.

The solution for the length of the cremaster muscle is plotted in Fig. 2 (solid blue line). The cremaster muscle grows to 5.25 mm in length when there is cell proliferation within the gubernacular bulb and cremaster muscle.

Table 1 Mean cell density (cells/mm^3) of (a) gubernacular bulb vs (b) proximal cremaster muscle during inguinoscrotal descent.

Region	Age (days)	(n)	Density (cells/mm^3)
(a) Bulb	2	3	30,019
	8	2	18,956
(b) Proximal Cremaster	2	3	35,529
	8	4	15,278

Table 2 Gubernacular length [$l(t)$](a), radius of cremaster at abdominal wall (b), cremaster muscle thickness (ie: width [$w(t)$])(c), and gubernacular bulb area and radius [$r(t)$](d) during inguino-scrotal descent.

Dimension	Age (days)	Mean (mm)	Range (mm)
(a) Length: $l(t)$	2	2.4	2.1–2.7
	8	4.3	3.5–5.1
(b) Radius cremaster sac at abdominal wall: $r(t)$	2	1.047	-
	8	2.0595	-
(c) Cremaster Muscle width : $w(t)$	2	0.135	0.120–0.150
	8	0.099	0.100–0.098
		Area (μm^2)	Radius : $r(t)(\mu m)$
(d) Gubernacular Bulb area	2	245,500	279.7
	8	47,500	123.0

When proliferation in the gubernacular bulb is switched off, there is little difference between this solution for the length of the cremaster muscle (Fig. 2, solid red line) and the full solution. By day 10 there is 0.20 mm difference. However when proliferation in the muscle is switched off (Fig. 2, dashed blue line), there is a pronounced difference in length of the cremaster muscle at day 10: it is approximately half the length of the full solution (3.00 mm). In fact from day 2 to day 10, when there is no proliferation in the muscle, the cremaster muscle only grows 1.00 mm compared to the 3.25 mm when proliferation is allowed everywhere.

3. Discussion

In this mathematical model of the growth of the gubernaculum, we found that the length of the cremaster muscle does not change significantly when the cell proliferation within the gubernacular bulb is neglected. The opposite is true when the cell proliferation in the muscle is neglected. However experimental evidence has shown that the gubernacular bulb is integral to the successful growth of the gubernaculum. Therefore the model suggests that the role of the gubernacular bulb in the growth of the cremaster muscle is more complex than just exporting cells into the muscle. Perhaps there is some signalling between the gubernacular bulb and cremaster muscle that affects the mitotic rate within the muscle.

Table 3 Mitotic rates in gubernacular bulb vs proximal cremaster muscle (fraction of BUdR-positive cells), derived from Hrabovszky et al., 2002 [5] and Ng et al., 2005 [8].

Age (days)	Gubernacular Bulb ($\tilde{x} \pm$ SD)	Age (days)	Proximal Cremaster ($\tilde{x} \pm$ SD)
0	0.138 ± 0.039	0	0.087 ± 0.016
2	0.174 ± 0.030		
4	0.155 ± 0.022	3	0.082 ± 0.010
6	0.126 ± 0.029	7	0.082 ± 0.017
8	0.075 ± 0.029		
10	0.048 ± 0.017	10	0.054 ± 0.014

Gubernacular protrusion from the abdominal wall during the inguino-scrotal phase is an echo of earlier embryonic outgrowths, such as limb buds, the branchial arches and the genital tubercle [9]. Recent molecular studies of limb bud development show the same molecules are involved across phyla as widely divergent as arthropods and vertebrates [10]. This suggests that body-wall outgrowths are regulated throughout evolution by an ancient set of conserved genes that control a range of different appendages [11,12].

Recent studies show that the gubernaculum in rodents has many molecular properties that are similar to an embryonic limb bud. For example, the tip of the gubernaculum can be labelled with a lipophilic vital dye to reveal the undifferentiated mesenchymal bulb that behaves like a limb bud growth centre, or "progress zone" [3]. Indeed, the gubernacular tip of the neonatal rat can be excised and grafted just like a limb bud progress zone, consistent with it containing a growth centre [2]. In addition, the fetal mouse gubernaculum expresses Fgf10 and Hoxa10, which are 2 proteins with essential roles in the developing limb bud [4].

Limb bud morphogenesis has been studied for many decades, but the cellular basis of its distally orientated elongation is still not fully understood. Computer simulations show that the observed differential proliferation rates, with highest rates in the progress zone, play no significant role in elongation. The authors concluded that both theoretical evidence and empirical evidence suggest that elongation is achieved by directional cell activities, rather than a proliferation gradient [13].

The modelling of gubernacular growth suggests that it grows in the same way as a limb bud, and may elongate by the bulb producing signalling factors that regulate cell division in the adjacent cremaster muscle. The limitations of modelling mean that it is not possible to draw firm conclusions, although the fact that our modelling predicts the gubernaculum and limb bud grow similarly is intriguing.

In conclusion, mathematical modelling of the gubernaculum suggests that the bulb may provide some directional signals to regulate elongation, rather than a simple proliferation gradient. Elucidation of these factors may be helpful in working out the causes of failed elongation or aberrant migration that must occur in cryptorchidism.

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