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Effects of long-term castration on the smooth muscle cell phenotype of the rat ventral prostate

Eliane Antonioli, Alexandre Bruni Cardoso and Hernandes F. Carvalho

Department of Cell Biology, Institute of Biology, State University of Campinas (UNICAMP), Campinas SP, Brazil

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Running title: SMC phenotype modulation in the rat prostate

Correspondence to: Hernandes F Carvalho
Department of Cell Biology – UNICAMP
CP6109
13083-863 Campinas SP, Brazil
E-mail: hern@unicamp.br
Fax: 55 19 3521 6111
Tel.: 55 19 3521 6118

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ABSTRACT

Smooth muscle is an important component of the prostatic stroma. We have previously shown that, despite extensive morphological changes, smooth muscle cells (SMCs) of the rat ventral prostate preserve some differentiation markers 21 days after castration. In the present study we investigated whether the expression of SMC markers is preserved in the rat ventral prostate after long-term castration. Adult Wistar rats were castrated and sacrificed 100 days after surgery. The ventral prostate was processed for histology, stereology, immunocytochemistry (SMα-actin and SM-myosin heavy chain [MHC]), transmission electron microscopy (TEM), and RT-PCR (smoothelin, sm22, calponin). The prostate of castrated rats showed a significant weight reduction, corresponding to only 5.6% of the control. Stereology showed that SMCs occupied the same proportion of the prostate volume, but suffered a significant reduction in absolute volume (5.5% of control). The SMCs were retracted and showed a spinous outline. TEM revealed the presence of an abundant myofibrillar component, dense plaques and an external lamina in these cells. SMCs were reactive to antibodies against SMα-actin and SM-MHC and expressed mRNA for smoothelin, sm22 and calponin. The results confirm that rat prostatic SMCs are affected by androgen deprivation. Though showing marked phenotypic changes, these cells express SMC markers at the protein (SMα-actin and SM-MHC) and mRNA (smoothelin, sm22 and calponin) levels. These observations support the idea that SMCs may modulate their phenotype (contractile *vs*. synthetic) without changing the differentiation state.

1 **Introduction**

2

3 The prostate gland largely depends on androgens for its embryonic and
4 pubertal development and for the maintenance of adult activity (Isaacs et al, 1994).
5 Surgical or chemical castration causes a marked involution of the organ, which is
6 mainly attributed to a decrease in epithelial cell activity and apoptosis. The stroma is
7 also subjected to marked changes after androgen deprivation, occupying a growing
8 fraction of the organ volume. In the stroma, in addition to a reduction in blood
9 vessels, increased vascular permeability (Shabsigh et al, 1998; 1990), and
10 extracellular matrix reorganization, mainly related to elastic fibers (Carvalho et al,
11 1997), basement membrane (Carvalho and Line, 1996) and collagen fibers (Vilamaior
12 et al, 2000), the functions of cellular components are also reprogrammed. Among
13 these components, smooth muscle cells (SMCs) are prominently affected. Previous
14 studies have suggested that prostatic SMCs progressively dedifferentiate after
15 castration. This suggestion arose from the observation that the myofibrillar fraction is
16 reduced in favor of an increase in the volume fraction occupied by organelles of the
17 secretory pathway, indicating that these cells undergo a contractile-to-synthetic
18 phenotypic change (Zhao et al, 1992; Niu et al, 2001; 2003). It was also shown that
19 SMCs sequentially lose some molecular markers, mirroring their appearance during
20 morphogenesis, suggesting that the phenotypic modulation was coupled to
21 dedifferentiation (Hayward et al, 1996).

22 More recently, new markers have been proposed to be specific for SMCs
23 (Doevendans and van Eys, 2002). Using some of these markers, we have shown that,
24 in addition to extensive morphological changes including an increase in the organelles

25 of the synthetic secretory pathway (Vilamaior et al, 2005), SMCs exhibit many of the
26 morphological landmarks of differentiated cells and preserve smooth muscle myosin
27 heavy chain and smoothelin expression up to 21 days after castration (Antonioli et al,
28 2004). This finding led us to propose that the contractile-to-synthetic phenotypic
29 change does not involve dedifferentiation.

30 We further argued that the discrepancy between our results and those reported
31 by Hayward et al (1996) might be attributable to the length of the androgen
32 deprivation period (21 *vs*. 100 days) and/or the use of distinct differentiation markers
33 (myosin, vinculin, desmin and laminin *vs*. smoothelin and myosin heavy chain), and
34 then decided to investigate whether long-term castration results in the loss of these
35 newer and more specific markers of SMC differentiation. Pursuing this task, we
36 investigated the expression of smooth muscle alpha-actin, smooth muscle myosin
37 heavy chain (at the protein level) and smoothelin, sm22 and calponin (at the mRNA
38 level), as well as morphological, ultrastructural and stereological alterations, to
39 determine the effects of long-term androgen deprivation on the differentiation state of
40 SMCs in the rat ventral prostate. The results allowed us to confirm that, despite
41 showing morphological changes, SMCs preserve their differentiated state after long-
42 term castration.

43

44 **Materials and Methods**

45

46 Animals and Histological Processing

47 Fifteen 3-month-old male Wistar rats were used. Ten animals were subjected
48 to orchiectomy by means of a scrotal incision under chloral hydrate anesthesia.
49 Ventral prostates were removed 100 days after surgery. Five age-matched rats were

50 used as controls. Protocol was approved by the Committee of Ethics on Animal
51 Experimentation from the State University of Campinas (Protocol number 1223-1).

52 The ventral prostates were dissected out, weighed and immediately fixed by
53 immersion in 4% formaldehyde in phosphate-buffered saline for 24 h. The samples
54 were then washed, dehydrated, cleared in xylene, and embedded in Paraplast Plus
55 embedding medium for immunocytochemistry. Some fragments were partially
56 dehydrated and embedded in Leica historesin for general morphology. Stereology was
57 performed as described previously (Antonioli et al, 2004; Garcia-Florez et al, 2005).
58 Volume density (Vv) was calculated as the percent of points in Weibel´s grid falling
59 on SMC. The total SMC volume was calculated as product of volume density per
60 prostatic weight, considering the specific gravity of the prostatic tissue as 1.0
61 (Huttunen et al. 1981). The results were compared using the Student's two-sample *t*-
62 test.

63

64 Transmission Electron Microscopy

65 Tissue fragments (~1 mm^3) were processed for transmission electron
66 microscopy. In brief, the fragments were fixed for 24 h in a solution containing 0.25%
67 tannic acid and 3% glutaraldehyde in Millonig’s buffer, postfixed in 1% osmium
68 tetroxide for 1 h, and then incubated in 0.5% uranyl acetate in maleate buffer
69 overnight before dehydration in a graded acetone series and embedding in Araldite
70 (Polysciences Inc., Warrington, PA). Ultrathin sections were contrasted with lead
71 citrate. Analysis and documentation were carried out under a Leo 906 transmission
72 electron microscope.

73

74 Immunocytochemistry

75 Five-micrometer sections were mounted on silanized glass slides, dewaxed
76 with xylene and rehydrated in a decreasing ethanol series. Endogenous peroxidase
77 activity was blocked with 3% hydrogen peroxide in water for 30 min. Nonspecific
78 protein-protein interactions were blocked by incubation with 3% bovine serum
79 albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA) in Tris-buffered saline
80 containing 0.1% Tween 20 (TBS-T) for 1 h. Monoclonal antibodies against smooth
81 muscle α-actin (cat. A2547; Sigma Chemical Co.) and smooth muscle myosin heavy
82 chain (cat. sc-6956; Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:50
83 and 1:100, respectively, in TBS-T containing 1% BSA, and applied to sections for 1 h
84 at room temperature. After three 5-min washes with TBS-T, the sections were
85 incubated with a peroxidase-conjugated antibody against mouse polyvalent
86 immunoglobulins (Sigma Chemical Co.) diluted 1:100 in 1% BSA in TBS-T for 1 h.
87 Sections were washed again and peroxidase activity was developed with 3,3'-
88 diaminobenzidine, followed by counterstaining with methyl green, air drying and
89 mounting in Entellan (Merck, Darmstadt, Germany). For smooth muscle myosin
90 heavy chain immunocytochemistry, sections were pretreated with 0.4% pepsin in 0.01
91 N HCl at 37°C for 30 min before endogenous peroxidase blockade. The specimens
92 were observed under a Zeiss Axioskop microscope and photographed using Kodak
93 100 Proimage film.

94

95 RT-PCR

96 The prostates were dissected out, weighed and immediately homogenized with
97 a Polytron in the Trizol reagent (Invitrogen-Life Tecnologies, Carlsbad, CA, USA).
98 Total RNA was then extracted according to the instructions provided by the

99 manufacturer. The amount of RNA was determined by measuring the absorbance at
100 260 and 280 nm using a correspondence factor of 40. For cDNA synthesis, reverse
101 transcription was performed using SuperScript III reverse transcriptase (Invitrogen-
102 Life Tecnologies) for 60 min at 50°C and for 15 min at 70°C. PCR was carried out
103 with 150 ng cDNA in a final volume of 25 μL containing Taq Polymerase PCR
104 Master Mix (Promega Corporation, Madison WI) and 0.6 pmol of the following
105 primer sets (Invitrogen-Life Tecnologies, São Paulo, Brazil) under the following
106 reaction conditions: β-actin (f: 5'-TCACCCACACTGTGCCCATCTACG-3'; r: 5'-
107 CAGXGGAACCGCTCATTGCCAATGG-3'; annealing temperature 64°C; 30
108 cycles); smoothelin (f: 5'-GTCGACATCCAGAACTTCCTCC-3'; r: 5'-
109 CGCAGGTGGTTGTACAGCGA-3'; annealing temperature 94°C; 35 cycles)
110 (Rensen et al, 2002); calponin (f: 5'-GAAGATCAATGAGTCAACCG-3'; r: 5'-
111 CCTTGAGGCCATCCATGAAG-3'; annealing temperature 61.5°C; 30 cycles), and
112 SM22 (f: 5'-AGGTCTGGCTGAAGAATGGC-3'; r: 5'-
113 TTCAAAGAGGTCAACAGTCTGG-3'; annealing temperature 60°C; 30 cycles). The
114 size of the reaction products for β-actin, smoothelin, calponin and sm22 was 64, 440
115 (visceral) and 330 (vascular), 150 and 200 bp, respectively. PCR products were
116 analyzed by 2% agarose gel electrophoresis in TAE buffer and visualized by ethidium
117 bromide staining. The 50-bp DNA Step Ladder (Promega Corporation) was used as
118 marker.

119

120 **Results**

121

122 Table 1 shows the body and prostate weight and the effect of androgen
123 deprivation for 100 days on these parameters. There was no variation in body weight,

124 whereas prostate weight showed a 20-fold reduction. Accordingly, the relative weight
125 of the ventral prostate was reduced to just 5.5% of the control.

126 Androgen deprivation resulted in marked remodeling of the rat ventral
127 prostate. In addition to marked epithelial modifications, H&E staining revealed that
128 control SMCs were elongated and flattened against the epithelial basement membrane,
129 showing a slightly irregular outline. Following castration, shortening of the cell was
130 observed accompanied by a pleating of the cell surface, leading to a spinous aspect
131 (Figure 1A and B). The SMCs apparently lost the contacts with each other and
132 presented wider extracellular spaces. Usually, more than one layer of SMCs was
133 found below the epithelial structures after castration.

134 Stereological analysis showed that androgen deprivation for 100 days did not
135 result in any modification of the prostatic volumetric fraction (Vv) occupied by SMCs
136 (Table 2). However, considering the large reduction in prostatic weight, long-term
137 castration caused a significant decrease in absolute SMC volume, i.e., SMC volume
138 corresponded to 5.5% of that calculated for the age-matched control (Table 2).

139 Transmission electron microscopy revealed important aspects of the SMCs,
140 which were elongated and exhibited a slightly irregular outline (Figure 2A-C). In
141 cross-sections, the irregular outline of the cell nucleus, scarcity of organelles, dense
142 plaques, and the presence of an external (basal) lamina were noted (Figure 2B and D).
143 The SMCs were separated from the epithelium by a single fibroblast layer (Figure
144 2C). Androgen deprivation promoted marked SMC reorganization, the most
145 prominent being retraction of the cytoplasm which resulted in a marked irregular
146 outline. Myofilaments were the predominant element of the cytoplasm (Figure 2E).
147 Collagen fibrils were closely incrusted in the grooves of the SMC surface (Figure 2E-
148 G). Fibroblasts were also found to extend thin processes amongst the collagen fibrils

149 in the SMC infoldings (Figure 2F). The external lamina, subplasmalemmal dense
150 plaques and abundant caveoli, three major structural components of SMCs, were
151 preserved after castration (Figure 2G). Although most SMCs were separated from one
152 another, close contacts were mantained at certain points (Figure 2G).

153 In addition to the morphological and ultrastructural characterization of the
154 effects of long-term androgen deprivation, we also investigated the expression of
155 some SMC markers. Immunocytochemistry revealed that smooth muscle actin (Figure
156 1C-E) and myosin heavy chain (Figure 1F-H) were present in the SMCs of control
157 rats (Figure 1C and 1F, respectively) and that androgen deprivation for 100 days had
158 no effect on this expression pattern (Figure 1D and E, and 1G and H, respectively).
159 Moreover, RT-PCR showed that smoothelin, calponin and sm22 were expressed at the
160 mRNA level in the ventral prostate of control and castrated rats (Figure 3). The
161 expression of the visceral isoform of smoothelin (Figure 3B) was identified.

162

163 **Discussion**

164

165 SMCs exert a common contractile function throughout the body. These cells
166 differ in terms of contractility, part of them showing a phasic contraction pattern,
167 while the remanining ones are tonic. Furthermore, in contrast to cardiac and skeletal
168 muscle these cells do not terminally differentiate, i.e., they might be recruited for
169 further proliferation and, moreover, for performing different functions. These
170 complex patterns of functioning might result from the intricate embryonic origins of
171 SMCs (Gittenberger-de-Groot et al, 1999).

172 Vascular SMCs have received more attention than the prostatic ones.
173 However, prostatic SMCs are also important because of their central role in benign

174 prostatic hyperplasia (Shapiro et al, 1992) and because they seem to be important in
175 regulating epithelial function and behavior, including in cancer (Cunha et al, 1996;
176 Hayward and Cunha, 2000). The primary response of prostatic SMCs to castration is
177 related to the fact that they express the androgen receptor (Prins et al. 1991; Hayward
178 and Cunha, 2000).

179 We have previously examined the behavior of prostatic SMCs in castrated rats
180 and observed that these cells undergo extensive morphological changes (Antonioli et
181 al, 2004) and present an increase in secretory organelles (Vilamaior et al, 2005) after
182 androgen deprivation for 21 days. We have also shown that these cells express smooth
183 muscle-specific actin and myosin heavy chains plus smoothelin (Antonioli et al,
184 2004), suggesting that these cells undergo marked phenotypic modifications in
185 association with distinct functions (Vilamaior et al, 2000), but preserve their
186 differentiation state. This assumption is in perfect agreement with the idea of Owens
187 et al (2004) on vascular SMC phenotype modulation (or switching). On the other
188 hand, this proposal contradicts a previous report by Hayward et al (1996), who studied
189 the the differentiation of smooth muscle during prostatic development as well as
190 changes related to androgen deprivation and concluded that the modifications reported
191 for the latter reproduced the developmental acquisition of differentiation markers.
192 This discrepancy might be attributed to the use of different differentiation markers, as
193 well as to the investigation of different periods of androgen deprivation.

194 The present study was then idealized to reproduce the long-term castration
195 experiment of Hayward et al (1996) using the same markers as employed before
196 (Antonioli et al, 2004), in addition to a detailed morphological study of SMCs. We
197 showed that SMCs occupies a twice volume of the prostate in these aged animals as
198 compared to 90-day-old animals (Antonioli et al, 2004) and that they became

199 markedly atrophic after 100 days of androgen deprivation. Although SMCs occupied
200 the same volume fraction of the organ (~10%) with respect to the age matched
201 controls, they showed a marked reduction in the total volume, considering the extreme
202 reduction in prostatic weight. At the ultrastructural level, SMCs were readily
203 recognized by the presence of abundant myofilaments, the external lamina (basal
204 membrane) and subplasmalemmal dense plaques. These cells were also reactive to
205 anti-smooth muscle actin and anti-myosin heavy chain, in addition to expressing
206 smoothelin, sm22 and calponin. While the last two markers might reflect the presence
207 of vascular smooth muscle, the expression of the visceral (or urogenital) isoform of
208 smoothelin (Rensen et al, 2002) is probably restricted to prostatic SMCs.

209 It is possible that a fraction of the SMC do indeed dedifferentiated and
210 contribute to the reduction in the total SMC volume in the prostate of castrated
211 animals, beside the death of some of them and the atrophy of the remaining
212 differentiated cells. This question will require precise methods for labeling and
213 counting SMC for different periods after castration.

214 It is not well known to what extent prostatic SMCs are comparable to vascular
215 SMCs. However, it has been shown for the latter that the quiescent (and contractile)
216 state is due to the expression and activation of CREB and that the transition to a
217 proliferative and migratory phenotype involves CREB inactivation (Reusch et al,
218 2004). Other transcription factors probably involved in this transition are GATA-6
219 and gax (favoring the quiescent state) and BTEB2 and Egr-1 (favoring the
220 proliferative state), while SRF and MEF2 would have dual effects in SMC behavior
221 (Walsh and Takahashi, 2001). Myocardin is another important factor regulating the
222 expression of SMC-specific molecules (Owens et al, 2004). Furthermore, the specific
223 arrangement of CArG elements [CC(AT)$_6$GG motif] and their variations within the

224 promoter region of genes encoding sm22, myosin heavy chain and α-actin are likely
225 to be responsible for setting the cell type specificity and temporal expression of SMC
226 specific genes (Owens et al, 2004). The expression and function of these transcription
227 factors have not been investigated in prostatic SMCs and thus deserve future study.

228 Another important question in the biology of prostatic SMCs is whether the
229 alterations in SMC phenotype observed after androgen deprivation are comparable to
230 those seen in cancer invasion. Researchers have studied the modifications in SMC
231 behavior during stromal activation in response to cancer progression and emphasized
232 that SMCs undergo progressive dedifferentiation to myofibroblasts (Cunha et al,
233 1996; Tuxhorn et al, 2001; 2002; Wong and Tam, 2002). Whereas the use of cell
234 cultures is of limited applicability since the mere placement of SMCs in culture is
235 sufficient to promote phenotypic changes, the lack of appropriate experimental
236 models limits the progress in this area.

237 In conclusion, we demonstrated that prostatic SMCs undergo phenotypic
238 modulation (or switching) upon androgen deprivation, while preserving major
239 differentiation markers. It should be emphasized that the phenotypic modulation of
240 SMCs upon androgen deprivation cannot be seen as a passive response but should be
241 considered to be an active adaptation to the new hormonal condition as well as to the
242 changing stromal (and organ) microenvironment, with possible contributions of
243 altered levels of TGF-β and bFGF (Niu et al, 2003).

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245

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321 **Figure legends**

322

323 Figure 1**.** H&E staining of a historesin section and immunocytochemistry for smooth
324 muscle actin and myosin heavy chain. H&E staining of the ventral prostate of control
325 animals **(a)** showed elongated smooth muscle cells (smc) usually present as a single
326 layer below the epithelium (Ep), with apparent contacts between each other. In
327 contrast, in castrated rats, the smooth muscle cells were markedly retracted and
328 atrophic, showing a spinous aspect with very thin processes and forming multiple
329 layers below the epithelium, and were mainly separated from each other by the
330 presence of extracellular matrix **(b)**. Smooth muscle actin **(c-e)** and myosin heavy
331 chain **(f-h)** were detected in smooth muscle cells of control **(c and f)** and castrated rats
332 **(d,e and g,h)**. Whereas the staining for actin was more diffuse throughout the cells,
333 that for myosin heavy chain was more concentrated in some regions of the cell,
334 especially around the nucleus. Bars = 10 μm.

335

336 Figure 2**.** Transmission electron microscopy of smooth muscle cells (SMCs) in the
337 ventral prostate of control and castrated rats. SMCs are flat and elongated, showing a
338 slightly undulated outline in non-castrated rats **(a)**. In cross-sections, further
339 morphological aspects such as the irregular outline of the cell nucleus, predominance
340 of myofibrils in the cytoplasm, and the presence of dense plaques and an external
341 lamina are readily observed **(b)**. In control animals, the cells were very elongated and
342 separated from the base of the epithelium by a single fibroblast layer **(c)**. The detail in
343 **d** shows the presence of an external lamina (asterisks) surrounding the SMCs and the
344 subplasmalemmal dense plaques (arrows), as well as abundant caveoli. In castrated
345 rats, the SMCs are retracted, exhibit a highly folded surface, contributing to the

346 spinous aspect, and a cell nucleus preserving the irregularity of the nuclear surface.
347 Collagen fibrils are intimately associated with the grooves of the cell surface **(e)**. The
348 detail in **f** shows that, in addition to collagen, fibroblast processes dig deep into the
349 grooves on the SMC surface. Although usually separated, the SMCs of castrated rats
350 **(g)** show some points of cell-cell adhesion (arrow). EC = endothelial cell; Ep =
351 epithelium; F = fibroblasts and/or fibroblast processes; smc = smooth muscle cell; col
352 = collagen fibrils; M = mitochondria. Bars: **a** = 10 μm; **b** = 2 μm; **c**, **e** and **g** = 5 μm; **d**
353 = 0.5 μm; **f** = 1 μm.
354
355 Figure 3**.** RT-PCR using mRNA extracted from the prostate of control and castrated
356 rats. The integrity of the cDNA preparation was confirmed with a primer set for β-
357 actin. A 64- bp amplicon was identified in control and castrated rats and in the urinary
358 bladder. The reaction for smoothelin amplified two bands, a vascular (430 bp) and a
359 visceral (330 bp) isoform from both control and castrated animals. The same was
360 observed for calponin and sm22, which were detected in control and castrated rats,
361 and presented amplicons of 150 bp and 200 bp, respectively. Smoothelin mRNA was
362 identified even 100 days after castration. **(Ct)** control; **(Cs)** 100 days after castration;
363 **(Bld)** bladder.

Table 1. Body and prostatic weight variation 100 days after castration (mean ± 1SD).

	Control (n=5)	**Castrated** (n=10)
Body weight (g)	449.0 ± 36.3	420.5 ± 35.9
Ventral prostate weight (g)*	0.421 ± 0.046	0.021 ± 0.006
Relative weight[1],*	0.089 ± 0.016	0.005 ± 0.002
% Variation	----------	-94.4

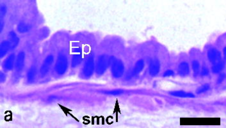
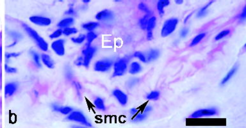
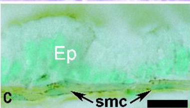
*Statistically significant at $p<0.001$.

[1]Calculated by dividing prostatic weight by total body weight.

Table 2. Stereological analysis of smooth muscle cells in the rat ventral prostate 100 days after castration (mean ± 1SD).

	Control	**Castrated**
Vv (%)	9.92 ± 2.70	11.05 ± 3.67
Absolute volume (mLx10^{-2})	4.18 ± 1.13	0.23 ± 0.08*

*$p<0.001$


Ep
smc
a

Ep
smc
b

Ep
smc
c

Ep
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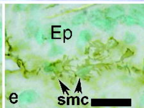
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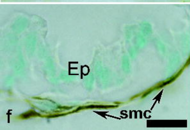
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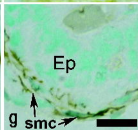
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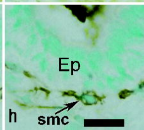
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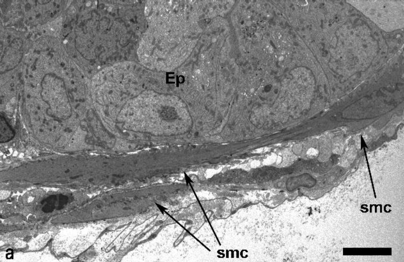
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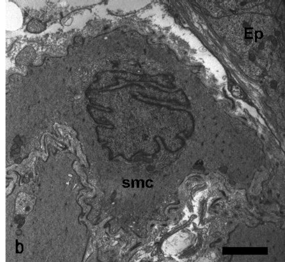
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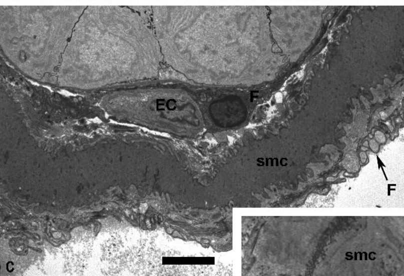
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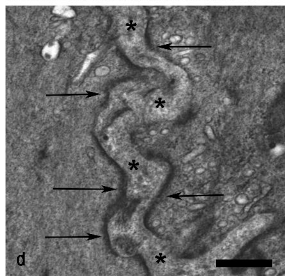
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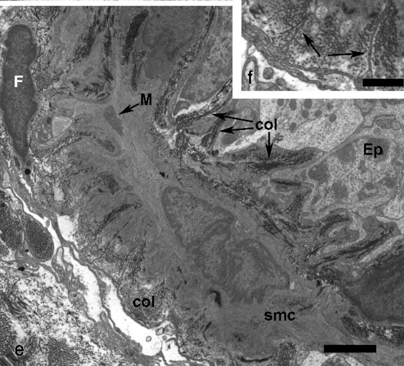
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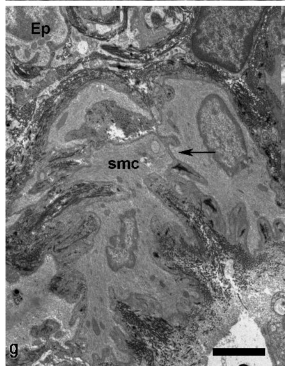
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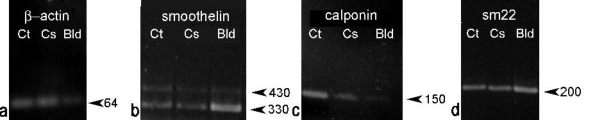

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