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TAIZE MACHADO AUGUSTO

"Modulação da expressão da heparanase-1 na próstata ventral de ratos e sua relação com aspectos gerais da fisiologia do órgão na castração e no *imprinting* estrogênico"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientador: Prof. Dr. Hernandes Faustino de Carvalho

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BANCA EXAMINADORA

Prof. Dr. Hernandes Faustino de Carvalho (Orientador) _____
Assinatura

Prof. Dr. Sérgio Luis Felisbino _____
Assinatura

Prof. Dr. Claudio Chrysostomo Werneck _____
Assinatura

Profa. Dra. Maria Aparecida da Silva Pinhal _____
Assinatura

Profa. Dr. Ruy Gastaldoni Jaeger _____
Assinatura

Prof. Dr. Paulo Pinto Joazeiro _____
Assinatura

Profa. Dra. Cristina Pontes Vicente _____
Assinatura

Profa. Dra. Leny Toma _____
Assinatura

Dedicatória

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ÍNDICE

LISTA DE ABREVIATURAS

AR - Androgen Receptor

ARE- Androgen responsive elements

BPA – Bisphenol A

BPH - Benign Prostatic Hyperplasia

cAMP - Cyclic Adenosine Monophosphate

CBP/p300 - CREB Binding Protein/ p300

CRE- cAMP-response-element

CREB - cAMP-response element-binding protein

CS- Chondroitin sulfate

DHT- Dihydrotestosterone

DS- Dermatan sulfate

E2 - Estrogen

ECM- Extracelular Matrix

EGR1- Early Growth Response Protein 1

ER - Estrogen Receptor

ERα - Estrogen Receptor α

ERβ - Estrogen Receptor β

Ets - E-twenty-six (transcription factor)

FGF - Fibroblast Growth Factor

FGF-2 - Fibroblast Growth Factor 2

GABP - Gamma-aminobutyrate transporter binding protein

GAG - Glycosaminoglycans

HA - Hyaluronic Acid

HDAC - Histone deacetylases

HPSE-1 - Heparanase-1

HPSE-2 - Heparanase-2

HS- Heparan sulfate

HSP - Heat shock Protein

IL-6R - Interleukin receptor 6

ISRE - Interferon-sensitive responsive element

MAPK - Mitogen-activated protein kinase

MAPK/ERK - Mitogen-activated protein kinase/ Extracellular signal-regulated kinases

MEK 1/2 - MAP ERK kinase 1/2

MMPs - Matrix metalloproteinases

NO - Nitric Oxide

P - Phosphorilation

Pi3K - Phosphoinositide 3-Kinase

PIN - Prostatic Intraepithelial Neoplasia

PKA - Protein Kinase A

PPARγ - Peroxisome Proliferator-Activated Receptor γ

PSA - Prostatic Specific Antigen

Sp1 - Specificity Protein 1 (transcription factor)

TGF-β- Transforming Growth Factor β

VEGF - Vascular Endothelial Growth Factor

RESUMO

A próstata é uma glândula do aparelho reprodutor importante na reprodução, sendo foco de várias afecções, dentre elas o carcinoma prostático. Ela é altamente dependente de testosterona e modulada por estrógenos, que desempenham papel fundamental no seu crescimento. Altas doses de estrógeno aplicadas em ratos no período neonatal causam efeitos irreversíveis na próstata quando na idade adulta, sendo o mais marcante o comprometimento do seu desenvolvimento e crescimento. A este efeito foi dado o nome de *imprinting* estrogênico. O estrógeno interfere no eixo hipotalâmico–hipofisário–gonadal bloqueando a produção de gonadotrofinas e inibindo a secreção da testosterona, mas também age localmente via receptores de estrógeno presentes na próstata.

A castração cirúrgica (pela retirada dos testículos) ou farmacológica acarreta involução da glândula prostática, efeito este que pode ser revertido pela reposição da testosterona. Durante este processo de regressão ocorre uma remodelação tecidual que em parte é dada pela morte das células epiteliais e pela reorganização da ECM subadjacente. Sabe–se da existência de vários fatores que atuam coordenadamente na reorganização da ECM, como enzimas proteolíticas, as MMPs, e a HPSE–1 (endo–β–glicuronidase, que degrada cadeias de HS).

Este trabalho foi dividido em dois grandes grupos de experimentos: no primeiro grupo, buscou–se investigar os efeitos globais do *imprint* estrogênico sobre o funcionamento prostático, a expressão da HPSE–1 na próstata ventral de ratos; no segundo grupo foi enfatizada a análise da contribuição da HPSE–1 na remodelação tecidual prostática que ocorre após a castração cirúrgica.

Para a identificação e avaliação do efeito do *imprinting* estrogênico sobre o metabolismo prostático e sobre a expressão da HPSE–1, foram realizados experimentos em ratos *Wistar* neonatos e posteriores avaliações das análises morfológicas, bioquímicas e moleculares de suas próstatas na idade adulta.

Neste trabalho pudemos demonstrar que a HPSE–1 desempenha papel relevante na segunda onda de morte das células epiteliais da próstata em regressão após a castração, com sua expressão aumentada 24 horas antes do segundo pico apoptótico da próstata em regressão. Pudemos evidenciar ainda que a exposição a altas doses de estrógeno na idade neonatal regula negativamente o estado metabólico prostático na idade adulta e está associado à diminuição da expressão gênica global como: maior compactação da cromatina, reduzida atividade nucleolar, e inibição da síntese protéica nas células epiteliais prostáticas. Este *imprinting* estrogênico resultante também refletiu no bloqueio da expressão da HPSE–1, o que foi confirmado tanto em nível de proteína como no de RNA mensageiro, particularmente nas células epiteliais. Estes resultados sugeriram um bloqueio em nível transcricional, por metilação do DNA.

ABSTRACT

The prostate gland is part of the male reproductive system and it is important for reproduction, and site of several diseases, including prostate cancer. It is highly dependent on testosterone and is modulated by estrogen, which play key roles in its growth.

High doses of estrogen administrated in neoborn rats cause irreversible effects in adult prostate, the most markedly the impairment of its development and growth. This effect is named estrogen *imprinting*. Estrogen interferes with the hypothalamic–pituitary–gonadal axis blocking the production of gonadotropins and thereby inhibiting testosterone secretion, besides acting locally via estrogen receptors found in the prostate.

Surgical (removal of the testis) or pharmacological castration, leads to the involution of the prostate gland, an effect that can be reversed by testosterone replacement. During this process, the tissue remodeling is partly given by epithelial cell death and reorganization of subadjacent ECM.

Several factors act in coordination for the reorganization of the ECM, such as proteolytic enzymes, the MMPs, and HPSE–1 (endo–β–glucuronidase that degrades HS).

This work was divided into two main groups of experiments: in the first group, we sought to investigate the global effects of estrogen *imprint* on the functioning prostate, and included analysis of the expression of HPSE–1 in rat ventral prostate, and in the second group we emphasized the analysis of contribution of HPSE–1 in prostatic tissue remodeling that occurs after surgical castration.

For the identification and evaluation of the effect of estrogen *imprinting* on metabolism of the prostate and on the expression of HPSE–1, experiments were performed

in rats and subsequent evaluations of newborns (morphological, biochemical and molecular features) were made in their adult prostates.

In this work we demonstrated that HPSE–1 plays a key role in the second wave of prostate epithelial cell death after castration, with its expression increased 24 hours before the second peak of apoptosis in prostate remodeling. We observed that chronic high doses of estrogen in the neonatal life regulates negatively the metabolic state of the prostate in adulthood and it is associated with the global gene expression decrease as: higher chromatin condensation, reduced nucleolar activity and inhibition of protein synthesis in the prostate epithelial cells. This estrogenic imprinting also reflected in blocking the HPSE–1 expression, which was confirmed at protein and mRNA synthesis blocking, particularly in epithelial cells. These findings suggested this blocking in a transcriptional level by DNA methylation.

1. INTRODUÇÃO

1.1 A GLÂNDULA PROSTÁTICA

A próstata é uma glândula acessória do aparelho reprodutor e a sua presença é universal nos mamíferos (Price, 1963) sendo responsável pela produção e armazenamento do líquido prostático, eliminado juntamente com os demais componentes do sêmen durante a ejaculação. Esse líquido contribui com o aumento da mobilidade e fertilidade dos espermatozóides e na neutralização da acidez da vagina, assumindo assim um papel importante no processo da fertilização. Há uma considerável variação na anatomia, bioquímica e patologias relacionadas à próstata entre as espécies de mamíferos. Nos humanos, os tecidos acessórios sexuais produzem altas concentrações de muitas substâncias biologicamente ativas que aparecem no sêmen, como frutose, ácido cítrico, espermina, prostaglandinas, zinco, proteínas que incluem as imunoglobulinas e enzimas específicas como proteases, esterases e fosfatases (Guyton, 1984, Garcia–Florez & Carvalho, 2005).

A existência de várias complicações patológicas que afetam essa glândula (prostatites, alterações proliferativas benignas e malignas), intensificadas com o envelhecimento, é o principal agente motivador do estudo dos mecanismos referentes à regulação do crescimento e da fisiologia prostática (Pfau *et al*., 1980).

Hoje o carcinoma prostático é uma das mais importantes doenças malignas diagnosticadas nos homens, principalmente a partir dos 50 anos de idade, sendo superado apenas pelo câncer de pulmão como causa de morte não acidental. Segundo o *National Cancer Institute* em 2010 nos Estados Unidos *(http://www.cancer.gov/cancertopics /types /prostate),* o número de mortes causadas pelo câncer de próstata foi calculado em 217.330 casos diagnosticados, enquanto no Brasil, segundo o Instituto Nacional de Câncer

(http://www.inca.gov.br/estimativa/2010/ index.asp?link=tbregioes_consolidado. asp&ID=1) foi calculado em 52.050 casos. Ao contrário de alguns tipos de tumores, a incidência do câncer de próstata tem aumentado ao longo dos anos. Isto está associado a dois fatores principais: maior presteza dos métodos diagnósticos e ao aumento da longevidade dos homens, uma vez que o câncer de próstata tem crescimento lento, como mencionado acima, e maior incidência com a idade dos indivíduos. A origem do câncer de próstata ainda é desconhecida, mas presume–se que vários componentes possam influenciar seu desenvolvimento, dentre os quais se destacam fatores genéticos, hormonais, dieta alimentar, além de fatores ambientais, também alvos de investigações.

O tratamento do câncer de próstata é muito controverso, pois são muitas as variáveis, como a idade do paciente, níveis de PSA e o estágio do tumor. Pacientes em condições inoperáveis devido à idade são tratados com terapia hormonal ou radiação. A terapia hormonal mais comum para o câncer de próstata é a privação androgênica, uma vez que a próstata é uma glândula altamente dependente de andrógeno e a maioria dos tumores de próstata origina–se das células epiteliais glandulares dependentes de andrógenos da região periférica da próstata (Cunha *et al*., 1987).

1.2 ANATOMIA PROSTÁTICA

A próstata humana é constituída por estruturas glandulares e não glandulares que se encontram reunidas sob uma mesma cápsula (McNeal, 1990) (Fig. 1). As estruturas glandulares túbulo–alveolares ramificadas estão imersas em um estroma denso, altamente irrigado e entremeado por fibras musculares lisas (Ham & Cormack, 1991). Já a próstata de roedores é lobulada e dividida através de sua estrutura anatômica e morfológica em: próstata ventral, lateral, dorsal e anterior (Hayashi *et al.*, 1991) (Fig. 1).

A próstata ventral é dividida em dois lobos, que são compostos por um conjunto de estruturas epiteliais túbulo–alveolares que se encontram envolvidas pelo estroma (Aümuller, 1979). Cada lobo constitui–se de um sistema de oito conjuntos de ductos que se ramificam distalmente. Esses dutos podem ser arbitrariamente divididos em segmentos proximais, intermediários e distais, conforme a distância das suas regiões com relação à uretra (Lee *et al.,* 1990; Shabisigh *et al.*, 1999), que diferem quanto aos tipos celulares (Cunha *et al.*, 1987), padrão de dutos (Hayashi *et al.*, 1991), tipo de secreção (Prins, 1991), resposta a hormônios (Prins *et al.*, 1991; Banerjee *et al.*, 1995), padrão de expressão gênica (Takeda *et al.*, 1990) e de síntese protéica (Lee *et al.*,1990; Prins *et al.*, 1991; Banerjee *et al.*, 1993). As diferenças fenotípicas das células epiteliais encontradas ao longo dos ductos prostáticos estão relacionadas com a distribuição diferenciada do tecido fibroso e muscular liso (Lee *et al.*, 1990; Sensibar *et al.*, 1991; Nemeth & Lee, 1996). Este último ocupa 5% do volume total da glândula e cerca de 14% do estroma (Antonioli *et al.*, 2004), apresentando papel preponderante nos mecanismos de estimulação parácrina sobre o epitélio (Farnsworth, 1999), na contratilidade do órgão e na hiperplasia prostática benigna.

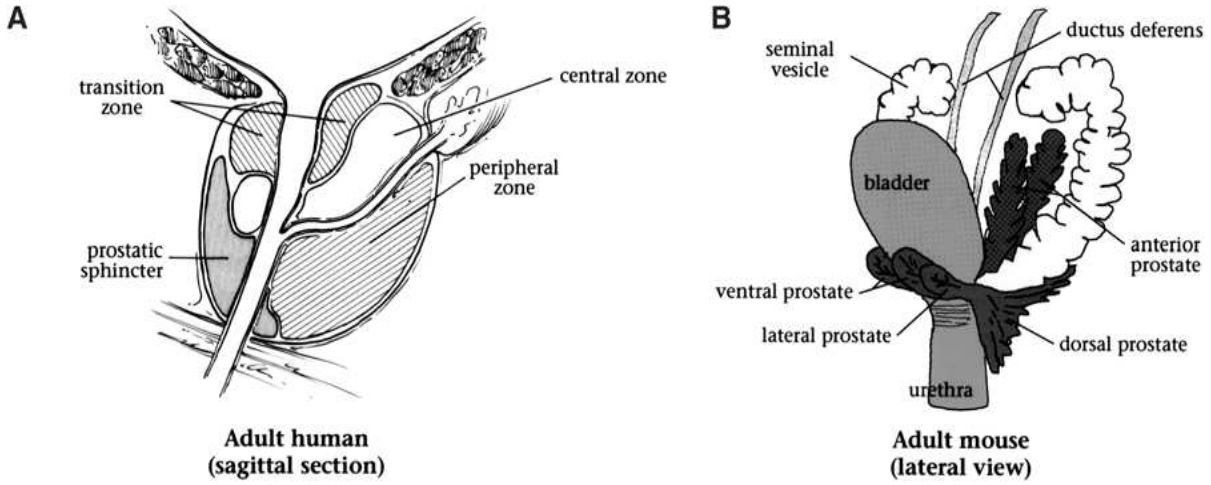

Figura 1 – *Ilustração esquemática da anatomia da próstata humana (A) e de camundongo (B).*

(Adaptados de McNeal, 1969 e de Cunha et al., 1987, respectivamente).

Outros tipos celulares também são encontrados no estroma prostático, dentre os quais os mastócitos, os fibroblastos, as células endoteliais e os pericitos, ao lado de terminações nervosas e gânglios sensitivos. Cada célula desempenha um papel importante e específico na manutenção e função secretora na próstata ventral. Como em outras glândulas, a atividade das células epiteliais é fortemente influenciada pelos componentes estromais, e as diferenças fenotípicas das células epiteliais encontradas ao longo dos ductos prostáticos parecem estar relacionadas com a distribuição diferenciada do tecido fibroso e muscular liso (Fig. 2) (Lee *et al.*, 1990; Sensibar *et al.*, 1991; Nemeth & Lee, 1996).

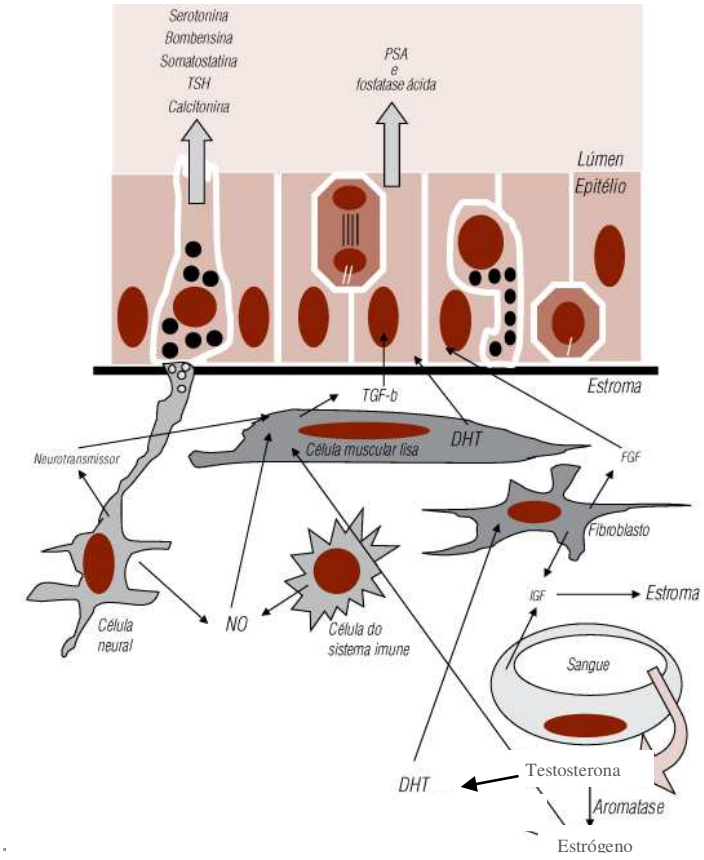

Figura 2 – *Complexas inter–relações entre o compartimento estromal e epitelial prostático.*
TGF–β (Transforming Growth Factor β), DHT (Dihydrotestosterone), NO (Nitric Oxide), FGF (Fibroblast Growth Factor), PSA (Prostatic Specific Antigen).

(Reproduzido de Taboga et al., 2009).

1.3 REGULAÇÃO HORMONAL PROSTÁTICA

Os andrógenos possuem um papel central na biologia da glândula prostática, contribuindo para o seu desenvolvimento e função da glândula adulta (Cunha, 1992), isto justifica as terapias anti–androgênicas para o tratamento do câncer prostático primário.

O desenvolvimento e funcionamento prostático também são modulados diretamente por hormônios somatotróficos (como insulina, prolactina e hormônio do crescimento), ácido retinóico e estrógenos (Webber, 1981; Prins, 2001), além das intrincadas interações epitélio–estroma (Lee, 1990), o que torna bastante complexo o mecanismo da regulação da fisiologia prostática.

Os AR e os ER são responsáveis pela mediação dos efeitos fisiológicos dos andrógenos e estrógenos respectivamente (Gelmann *et al.,* 2002; Sasaki *et al.,* 2003). O AR atua fundamentalmente como fator de transcrição (Fig. 3). Resumidamente o receptor localizado no citoplasma se liga à testosterona ou à diidrotestosterona, dissocia–se de uma proteína HSP, dimeriza–se, e é translocado para o núcleo, onde, em conjunto com uma série de co–ativadores e co–repressores, ativa ou inativa diferentes conjuntos de genes (Heinlein e Chang, 2002). O AR clássico tem 110 KDa e possui várias características em comum com os membros da família dos receptores nucleares, como os receptores de estrógeno, de progesterona, dos hormônios da tireóide e com os PPAR (Jacobs *et al.,* 2003). A figura 4 representa a estrutura gênica e o mecanismo de ativação do receptor de andrógeno.

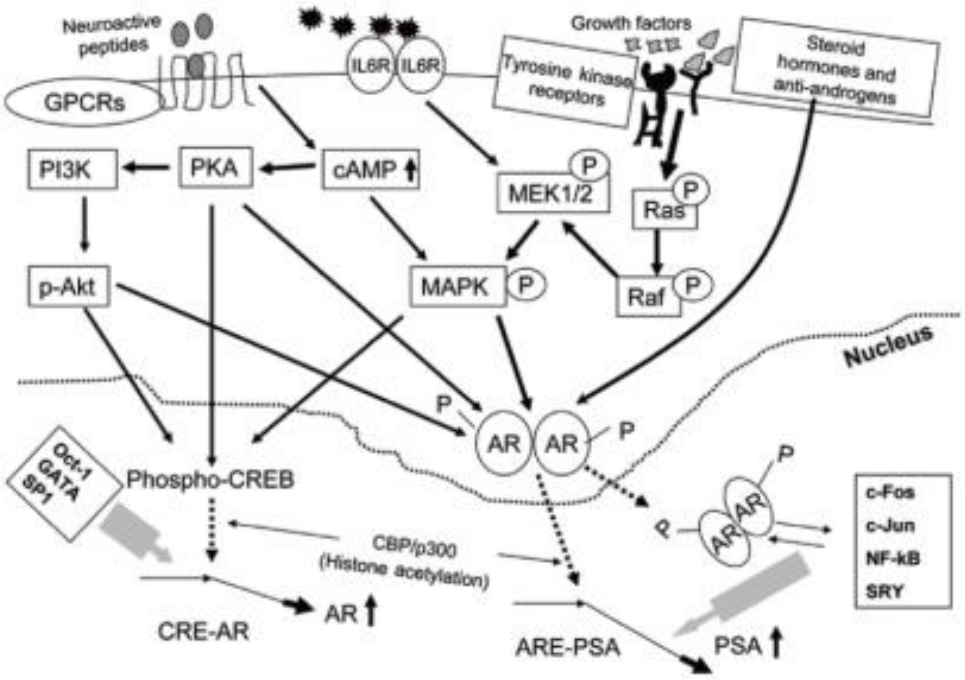

Figura 3 – *Vias de sinalização citoplasmáticas e nucleares mediadas pelo receptor de andrógeno (AR). Diagrama simplificado das interações entre fatores de crescimento, citocinas e peptídeos neuroativos com seus receptores que levam à ativação de quinases citoplasmáticas (PKA, PI3K) e segundos mensageiros (cAMP), seus efetores downstream da cascata de sinalização, co–ativadores e repressores. Similar às moléculas esteróides e alguns anti–andrógenos, o seu efeito em rede é a translocação nuclear do AR fosforilado. Como co–ativador, a CBP/p300 guia a sinalização mediada pelo AR com vias de sinalização ativadas por outros moduladores de crescimento.*

(reproduzido de Koochekpour et al., 2010)

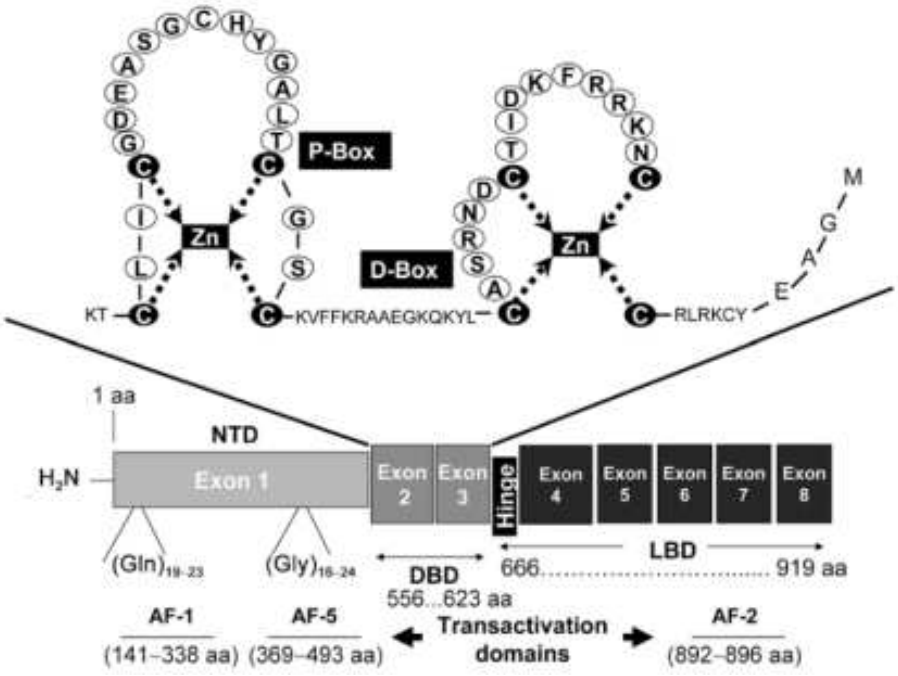

Figura 4 – *Esquema da estrutura do receptor de andrógeno (AR) com seus dois zinc fingers. Os éxons, três domínios funcionais e as posições relativas das repetições de poliglutamina (Gln22) e poliglicinas (Gly24) na região N–terminal estão destacadas.*

(reproduzido de Koochekpour et al., 2010)

Assim como o AR, os ER pertencem à família dos receptores nucleares e apresentam dois subtipos, ERα e ERβ, que têm papéis fisiológicos distintos. Os dois receptores compartilham homologia entre si, mas são produtos de diferentes genes (Yang *et al*., 2003).

Os dois receptores de estrógeno ERα e ERβ estão presentes na próstata, sendo que no animal adulto o ERα é expresso predominantemente no estroma e o ERβ expresso principalmente no epitélio (Weihua *et al*., 2002). Foi demonstrado que o silenciamento do ER através da metilação do seu promotor está envolvido na hiperplasia prostática benigna e no desenvolvimento do câncer de próstata (Li *et al*., 2000).

A ação dos estrógenos sobre a morfogênese ductal e diferenciação celular prostática é complexa (Prins *et al*., 2001). Observa–se que uma breve exposição de roedores a estrógenos durante o desenvolvimento neonatal provoca efeitos irreversíveis e dose–dependentes na morfologia, organização celular e função prostática (Prins *et al*., 1992). A exposição a baixas doses de estrógeno durante a gestação de camundongos provoca aumento dos níveis de receptor de andrógeno, no brotamento ductal e no peso da próstata no animal adulto (Nonneman *et al*., 1992), enquanto que a exposição a altas doses compromete o crescimento prostático, gera defeitos na diferenciação epitelial, alterações na função secretora, aumento na incidência de PIN e prostatites (Prins, 1992; Prins *et al*., 2001). Esse efeito sobre a próstata causado pela administração neonatal de altas doses de estrógenos, é devido não somente a alterações nas concentrações de andrógeno via ações permanentes sobre o eixo hipotálamo–hipófise–gonadal, mas também a efeitos diretos na próstata, uma vez que a administração de testosterona não reverte os efeitos (Huang *et al*., 2004), recebe a designação *imprinting* estrogênico (Fig. 5).

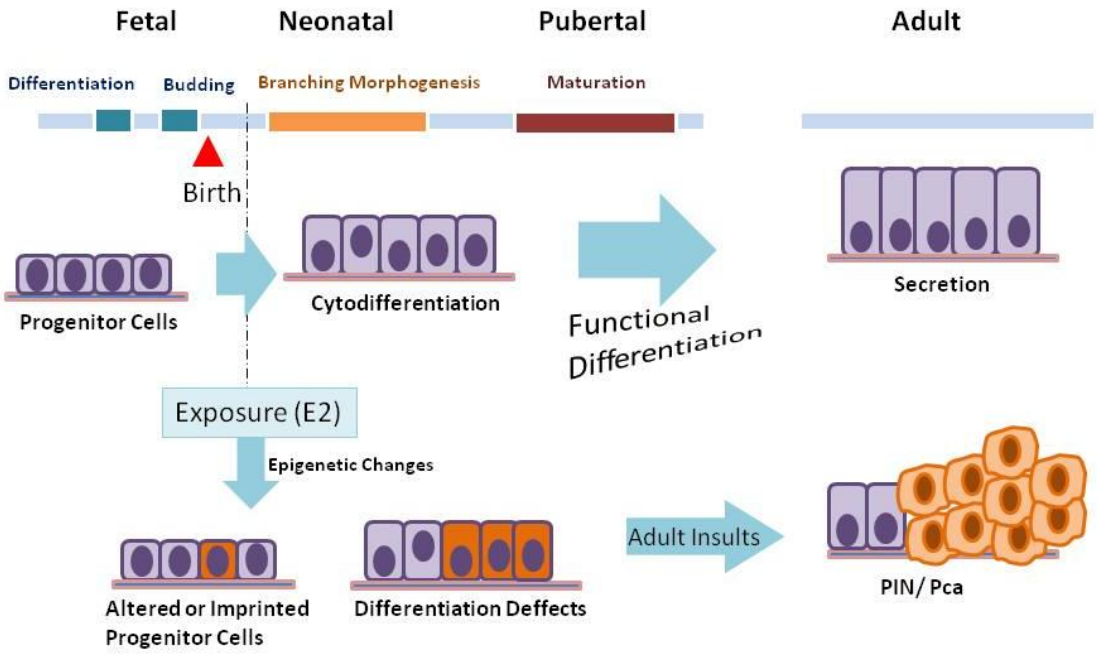

Figura 5 *– Modelo de regulação epigenética do desenvolvimento da glândula prostática decorrente da exposição neonatal ao estradiol, E2 ou um disruptor ambiental como o BPA.*

(adaptado de Prins et. al., 2007)

Altas doses de estrógenos administradas em animais adultos funcionam como uma castração química, resultando na inibição do eixo hipotalâmico–pituitária–gonadal pela supressão do hormônio liberador de gonadotrofinas e conseqüente diminuição da secreção do hormônio testosterona pelos testículos (Neubauer *et al.,* 1981; Thompson *et al.,* 1979). Esse efeito pode ser revertido (contrariamente àqueles administrados em neonatos) pela reposição de testosterona ou diidrotestosterona.

Já está bem estabelecido que alguns dos andrógenos circulantes são convertidos a estrógenos em vários tecidos periféricos pela enzima aromatase (Simpson *et al*., 1999). A

aromatase foi identificada na próstata humana, sugerindo que a glândula seja capaz de realizar a reação de aromatização e uma possível fonte local de estrógeno (Tsugaya *et al*., 1996).

Os estrógenos participam de várias mudanças patológicas na próstata sendo a mais bem descrita a indução da metaplasia escamosa relatada em várias espécies de mamíferos (Dore *et al.,* 2005; Risbridger *et al.,* 2001; Nevalainem *et al.,* 1993).

1.3.1 REGULAÇÃO HORMONAL PROSTÁTICA E SEUS EFEITOS SOBRE A MATRIZ EXTRACELULAR (MEC)

A mais marcante demonstração da dependência androgênica para manutenção da fisiologia prostática é revelada pela diminuição do peso da glândula e remodelação tecidual que se segue à privação androgênica (Bruni–Cardoso *et al.,* 2010). A regressão prostática seguida à castração resulta de vários fenômenos que incluem diminuição de vacúolos secretores (Heyns, 1990), aumento dos níveis de enzimas degradativas como ribonucleases (Engel *et al.*, 1980), alteração no sistema elástico (Carvalho *et al.*, 1997b), no colágeno VI (Carvalho *et al*., 1997a) e expressão de vários componentes que levam à apoptose, tais como a catepsina D (Sensibar *et al.*, 1991), o ativador de plasminogênio do tipo uroquinase (Freeman *et al.*, 1990) e variações na expressão de algumas MMPs, (Bruni-Cardoso *et al*., 2010; Justulin *et al*., 2010). As alterações da matriz extracelular também incluem variações no conteúdo dos GAGs. Kofoed *et al.* (1971) e Terry e Clark (1996) observaram que a atrofia prostática pós–castração causa alterações nos níveis qualitativos e quantitativos dos GAGs que podem ser revertidas pela reposição de andrógeno, e que estas alterações são mais pronunciadas na próstata ventral.

Neste lóbulo, foi demonstrado que o conteúdo de GAGs sulfatados por próstata caem cerca de 70% em resposta à castração. A administração de propionato de testosterona reverteu este efeito, sendo que o conteúdo de GAGs ultrapassou os níveis dos controles, indicando uma relação estreita entre o estímulo androgênico e o conteúdo de GAGs (Augusto *et. al.,* 2008; Fernandes, 2000; Terry & Clark, 1996).

Dados do nosso laboratório demonstraram que o DS é o GAG mais abundante da próstata. Sua predominância foi mantida após a castração, sendo ele o responsável pelo aumento na concentração dos GAGs em resposta à privação androgênica. A proporção de CS diminui após a castração e o conteúdo de HS aumenta, consistente com o acúmulo de membrana basal (Fernandes, 2000; Carvalho & Line, 1996). É importante mencionar que a discrepância entre o conteúdo total de cada glicosaminoglicano e a variação nas suas concentrações após castração deve–se ao fato de que há marcante redução do peso do órgão nesta condição de privação androgênica (Augusto *et al.,* 2008).

Uma das características de transformações malignas é a diminuição na biossíntese dos GAGs, principalmente das cadeias de HS (Timar *et al.,* 2002; Sanderson, 2001). Os tumores de origem epitelial são geralmente associados com aumento nos níveis de CS e de HA e diminuição de DS e HS (Iozzo *et al.*, 1981). A quantidade de CS encontra–se aumentada na próstata hiperplásica e em carcinomas, enquanto o conteúdo de HS e o seu grau de sulfatação parecem diminuir no carcinoma prostático (DeKlerk *et al.*, 1984). Os baixos níveis de HS de superfície celular também estão correlacionados com alta capacidade metastática de muitos tumores (Sanderson, 2001). Estes achados sugerem que mudanças nos níveis de HS estão relacionadas com o crescimento de tumores e com o grau de diferenciação celular. Outros estudos têm revelado que o aumento na concentração de condroitim sulfato tem relação com a

hiperplasia, enquanto o aumento na relação CS:DS está associado ao desenvolvimento de tumores malignos (Iida *et al.*, 1997).

1.3.2 A HEPARANASE–1 E SEUS EFEITOS SOBRE A MATRIZ EXTRACELULAR

Algumas evidências indicam que os proteoglicanos de HS agem na inibição da invasão celular pela promoção das interações célula–célula e célula–ECM, e por manter a integridade estrutural da ECM (Timar *et al.,* 2002; Sanderson, 2001).

Dois tipos principais de proteoglicanos de HS ligados à superfície celular foram identificados:

(i) *Syndecan* (proteoglicano transmembrana): possui quatro isoformas onde cadeias de HS ficam ancoradas no seu núcleo protéico no domínio extracelular, e cadeias de CS ancoradas próximo da superfície celular (Bernfield, 1999);

(ii) *Glypican l*igado ao glicosil–fosfatidil–inositol com seis isoformas, com várias cadeias de HS ancoradas perto da superfície celular e na extremidade do seu ectodomínio (Fransson, 2004).

Na ECM podem–se destacar outros dois tipos principais de proteoglicanos de HS:

(iii) *Agrin*, abundante na maioria das membranas basais, principalmente nas regiões sinápticas (Cole & Halfter, 1996);

(iv) *Perlecan*, com ampla distribuição tecidual, presente nas membranas basais e uma estrutura muito complexa e modular (Iozzo, 1998).

Augusto e colaboradores (2008) observaram que a regressão da próstata ventral de ratos causada pela castração está associada à diminuição no conteúdo de HS e aumento da atividade enzimática da HPSE–1.

A HPSE–1 é uma endoglicosidase que cliva cadeias de HS em sítios específicos. Esta atividade está relacionada à metástase, à neovascularização e a processos inflamatórios, uma vez que o HS é um componente importante das membranas basais (Bernfield *et al.*, 1991; Iozzo & San Antonio, 2001; Kjellen & Lindahl, 1991; Murdoch *et al.*, 1992). A HPSE–1 é preferencialmente expressa em tumores, sendo que sua super expressão em células tumorais confere um fenótipo invasivo (Ilan *et al.*, 2006).

A expressão da HPSE–1 que possui atividade enzimática é restrita à placenta, pele e células sangüíneas como plaquetas, neutrófilos, monócitos, mastócitos e linfócitos com pouca ou nenhuma expressão nas células do tecido conjuntivo (Vlodavsky & Friedmann, 2001; Vlodavsky *et al.*, 1992; Bartlett *et al.*, 1995; Dempsey *et al.*, 2000; Vaday & Lider, 2000; Bernard *et al.*, 2001; Parish *et al.*, 2001; Matzner *et al.*, 1985; Mollinedo *et al.*, 1997). Durante a embriogênese, a enzima é preferencialmente expressa em células do sistema vascular e nervoso (Goldshmidt *et al.*, 2001)**.**

Goldshmidt *et al.* (2003) sugeriram um possível envolvimento da HPSE–1 na interação da célula com a ECM, na transdução de sinais e na sobrevivência celular. Uma variedade de fatores de crescimento, como o FGF–2 e VEGF, além de citocinas, quimiocinas, e enzimas, pode ter seu potencial de ação aumentado pela atividade da HPSE–1, afetando profundamente a função celular e tecidual, o que foi demonstrado em particular para as células musculares lisas vasculares (Fitzgerald *et al.,* 1999; Elkin *et al.*, 2001).

Além da degradação de componentes protéicos da membrana basal, acredita–se que os fragmentos de HS sejam importantes para a liberação de fatores de crescimento ligados a ela (Ishai–Michaeli *et al.*, 1990; Bernfield *et al.*, 1991; Flaumenhaft *et al.*, 1992).

Apenas um gene para a Hpse–1 codifica a proteína com atividade enzimática (Hullet *et al.*, 1999; Kussie *et al.*, 1999; Toyoshima & Nakajima, 1999; Vlodavsky *et al.*, 1999). A análise da seqüência deste gene revelou que a Hpse–1 é altamente conservada em humanos, ratos, camundongos e outras espécies (Goldshmidt *et al.*, 2001; Parish *et al.*, 2001).

O cDNA para a Hpse–1 codifica uma proteína de 543 aminoácidos com massa molecular de ~ 65 KDa passando por processamento proteolítico no lisossomo onde são formados um fragmento de 8 KDa (correspondente à região N–terminal) e outro de 50 KDa (correspondente à região C–terminal) que compõem um heterodímero, para atuar como enzima ativa (Cohen *et al.*, 2005).

A expressão do gene da Hpse–1 depende de fatores de transcrição *Ets* (Lu *et al.*, 2003) e *Egr1* (DeMestre *et al.*, 2003), além do fator de iniciação de tradução eucariótico 4E (Yang *et al.*, 2003). Processos epigenéticos caracterizados principalmente pela hipermetilação de região promotora do gene da Hpse–1, podem levar à inibição do seu gene. Examinando uma série de linhagens de células derivadas de tumores foi encontrado que aquelas que exibiam a atividade da HPSE–1 tinham pelo menos um alelo não metilado. Em contraste, linhagens celulares que não exibiam expressão ou atividade da HPSE-1, como as células C6 de glioma de rato, apresentam todos os alelos metilados. Quando estas células foram tratadas com agentes demetilantes como a 5–azacitidina, a expressão do gene da HPSE-1 foi restaurada, acompanhada pelo aumento da capacidade metastástica *in vivo* (Shteper *et al.*, 2003).

Mecanismos regulatórios adicionais podem operar localmente ou de modo sistêmico na regulação transcricional da Hpse–1, sendo o 17–β–estradiol um importante exemplo de regulador sistêmico da tumorigênese. Elkin *et al.,* (2003) identificaram elementos responsivos ao estrógeno no promotor da heparanase e demonstraram sua funcionalidade através de ensaios com o gene repórter da luciferase dirigido pelo seu promotor, e pela inibição do receptor pela utilização de tamoxifeno (inibidor estrogênico utilizado convencionalmente em terapia endócrina de câncer de mama), ocasionando a inibição de expressão da Hpse–1.

Embora estas observações sejam relevantes para o câncer de mama, mecanismos que promovem a expressão da HPSE–1 em outros tecidos ainda são pouco entendidos.

Na BPH e em bexigas normais a freqüência de metilação da região promotora da HPSE–1 é maior do que em carcinomas. Observou–se uma correlação entre a expressão da HPSE–1 nos carcinomas de bexiga e de próstata e os níveis de expressão de EGR1, um fator implicado na transcrição da Hpse–1 (Ogishima *et al.*, 2005; DeMestre *et al.*, 2003 e 2005). Em contraste com outros fatores de transcrição como SP1 e Ets, associados com níveis basais de transcrição do gene da Hpse–1 (Jiang *et al.*, 2002), o EGR1 está relacionado com a transcrição induzida do gene da Hpse-1 (DeMestre *et al.*, 2003 e 2005). O EGR1 é rapidamente induzido em resposta a uma variedade de sinais como fatores de crescimento e citocinas, sendo aumentado em tumores humanos (Abdulkadir *et al.*, 2001) e fortemente implicado na angiogênese tumoral (Khachigian, 2004a).

Pouco se sabe sobre repressores relevantes na transcrição da Hpse–1. Sabe–se entretanto, que a *p53* inibe constitutivamente a transcrição pela ligação direta ao promotor da Hpse-1. Esta inibição envolve o recrutamento HDACs (Baraz *et al.*, 2006). A Figura 6 representa resumidamente alguns destes elementos de regulação de expressão da Hpse–1.

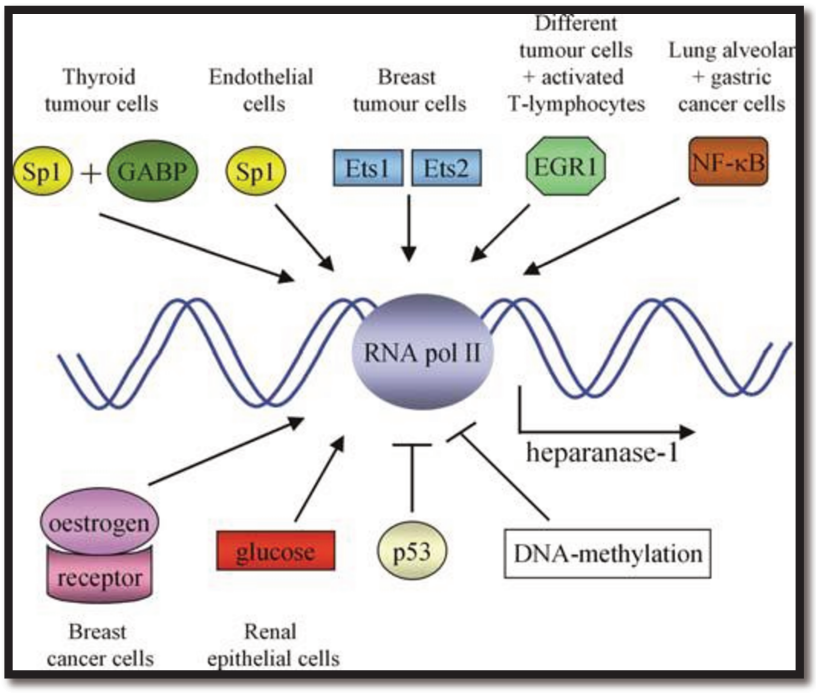

Figura 6 – *Regulação da expressão do RNAm da Hpse–1 em diferentes tipos celulares. A expressão do RNAm da Hpse–1 pela RNA polymerase II (RNA pol II) é positivamente regulado por fatores de transcrição como Sp1,GABP, Ets1 e Ets2, EGR1,NF–κB, estrógeno e glicose (ou fatores de transcrição induzidos pelos dois últimos). A expressão é negativamente regulada pelo fator de transcrição p53 e por metilação de DNA.*

(Vreys & David, 2007).

Análises computacionais do segmento de 1,9 Kb do promotor da Hpse–1 revelaram dois elementos responsivos consensos ISREs que especificamente ligam fatores de transcrição ativados pelo interferon (Edovitsky *et al.*, 2006). Estes resultados apontam o endotélio vascular como uma fonte celular importante da atividade enzimática da HPSE–1 que, por sua vez permite a remodelação da membrana basal vascular, aumentando a permeabilidade vascular e permitindo o extravasamento de células tumorais metastáticas, de leucócitos e de proteínas plasmáticas.

Há evidências de que alguns inibidores da HPSE–1 também inibem a função do HS. Acredita–se que a substância PI–88 compete com o HS pelo seu sítio de ligação em fatores de crescimento, bloqueando sinais que promovem a angiogênese (Khachigian & Parish, 2004b). Outros inibidores, como a suramina, bloqueiam a sinalização de fatores de crescimento pela competição com o HS endógeno (Coffey *et al.*, 1987; Marchetti *et al.*, 2003; Pesenti *et al.*, 1992).

Estudos indicaram também que a heparina inibe a metástase por bloquear as interações entre células tumorais e plaquetas, impedindo que os agregados de células tumorais se alojem na microvasculatura (Borsig *et al.*, 2001).

A HPSE–1 apresenta também uma proteína homóloga, conhecida como HPSE–2. Estudos descreveram que a HPSE–2 está envolvida na proliferação neoplásica, mas não exclusivamente associada com processos invasivos, uma vez que sua expressão não varia entre processos tumorais benígnos e malígnos (Moura *et al.,* 2009).

Recentemente, Levy–Adam *et al.,* (2010) descreveram evidências de que a HPSE–2c (uma das proteínas geradas por *splicing* alternativo da HPSE–2), possui também atividade enzimática, maior afinidade por heparina e HS e habilidade em se associar fisicamente com a HPSE–1, atenuando assim a função enzimática da HPSE–1 e conferindo uma sobrevida a pacientes com câncer de pescoço e pulmão.

Além da função enzimática da HPSE–1, esta também pode exibir atividades não enzimáticas. A presença da pró–enzima na superfície celular promove uma adesão celular firme, refletindo um papel nas interações célula–matriz extracelular (Goldshmidt *et al.*, 2003). A HPSE–1 intensifica a via de sinalização da Akt, que está relacionada à sobrevivência

celular e estimula a célula endotelial dependente de PI3K e de *p38* a migrar e invadir (Gingis–Velitski *et al.*, 2004).

A localização nuclear da HPSE–1 foi correlacionada com a manutenção da diferenciação celular, porém não se sabe se a transcrição gênica e a manutenção do estado celular diferenciado é devido à interação direta da HPSE–1 com o DNA ou é uma conseqüência da degradação do HS nuclear mediado pela heparanase (Ohkawa *et al.*, 2004).

Progressos no campo e a geração de ferramentas genéticas nos anos recentes (camundongos trangênicos e *knockout* para HPSE–1) levaram à descoberta de novos conceitos que expandem as inúmeras funções da HPSE–1 e sua significância na progressão tumoral e em outras patologias (Zcharia *et al.,* 2009; 2004, 2005a, 2005b). Apenas a título de exemplo, camundongos *knockout* se desenvolvem normalmente, são férteis e não exibem nenhuma anormalidade anatômica aparente (Zcharia *et al.,* 2009), apesar da completa falta de expressão da HPSE e de suas atividades enzimáticas neste animal.

A deficiência da HPSE–1 é acompanhada por marcante elevação dos níveis dos membros da família MMPs (–2, –9, –14) de uma maneira órgão–dependente. Por exemplo, nos camundongos *knockout* a expressão de MMP–14 no fígado é oito vezes maior quando comparado ao selvagem; e nas mamas a MMP–2 tem seu nível aumentado duas e meia vezes sugerindo que estas MMPs compensam a deficiência da HPSE-1 tecido–específica, sendo uma das razões para a super ramificação da glândula mamária nos camundongos *knockout* (Zcharia *et al.,* 2009).

Foi demonstrado que a ação de algumas MMPs pode facilitar e aumentar a atividade da HPSE–1 resultando numa mudança fenotípica em células musculares lisas vasculares (Fitzgerald *et al.*, 1999), e que a expressão da heparanase eleva os níveis de expressão de

MMP–9 (que atua na clivagem do *sindecan–1*), em comparação ao silenciamento da HPSE–1 que reduziu a atividade da MMP–9 (Purushothaman, 2008).

Dados do nosso laboratório evidenciaram que a inibição da Hpse–1 por pequenos duplexes de RNA (siRNA) diminui a expressão da MMP–2 no desenvolvimento prostático, sugerindo que a HPSE–1 e as MMPs atuam de forma coordenada na degradação da ECM (Barbosa *et al.*, dados preliminares).

Com a disponibilidade da HPSE–1 recombinante e o estabelecimento de métodos de *screening* em massa, uma variedade de moléculas inibitórias foram desenvolvidas, incluindo anticorpos neutralizantes, peptídeos, pequenas moléculas, espécies não anti–coagulantes de heparina, e várias outras moléculas polianiônicas, como laminarina sulfato, suramina e PI–88 (Ferro *et al.*, 2004; Simizu *et al.*, 2004). A inibição da HPSE-1 pela carragenina–λ resultou numa diminuição da formação de metástases, sugerindo uma efetiva contribuição da heparanase neste processo (Marchetti *et al.*, 1996).

2. JUSTIFICATIVA

Além da sua importância na função reprodutiva masculina, há crescente interesse na compreensão do desenvolvimento de doenças que acometem a próstata dada sua importância na função reprodutiva masculina, principalmente associada ao envelhecimento e às variações hormonais dela decorrentes. Parece–nos relevante investigar aspectos de regulação da fisiologia prostática como as influências hormonais e a contribuição da matriz extracelular para a compreensão do mecanismo de regulação da expressão e atividade de enzimas em processos relacionados à remodelação tecidual, que são importantes no desenvolvimento, em vários processos fisiológicos normais e na invasão tumoral. Existem evidências de que o estrógeno e compostos relacionados participam da regulação da fisiologia prostática e do processo de remodelação da ECM, estimulando e/ou inibindo a atividade do promotor do gene da Hpse–1, reforçando a necessidade de aprofundado enfoque no conhecimento dos mecanismos de regulação desta enzima na próstata, seja nos modelos experimentais correntemente em uso ou na proposição de novos modelos.

3. OBJETIVOS GERAIS

I) Avaliação da expressão da Hpse–1 na próstata ventral de ratos submetidos à castração cirúrgica e o estabelecimento do seu papel durante a remodelação tecidual da próstata ventral de ratos.

II) Avaliação dos efeitos globais devido ao tratamento neonatal com altas doses de estrógeno sobre o desenvolvimento e metabolismo da próstata ventral de ratos adultos.

III) Avaliação do efeito do *imprinting* estrogênico sobre a expressão da Hpse–1 na próstata ventral de ratos adultos.

4. RESULTADOS – APRESENTADOS EM FORMATO DE ARTIGO CIENTÍFICO.

Os resultados obtidos durante o desenvolvimento deste trabalho são apresentados em formato de artigos científicos publicados ou submetidos à publicação.

4.1 **MANUSCRITO I :** Submetido para *The Anatomical Record* (02/02/2011)

"HEPARANASE–1 ACTIVITY IS NECESSARY FOR THE SECOND WAVE OF EPITHELIAL–CELL DEATH IN THE RAT VENTRAL PROSTATE AFTER CASTRATION"

Autores: Taize M. Augusto, Henrique Pravatta, Alexandre Bruni–Cardoso, Danilo M. Damas–Souza and Hernandes F. Carvalho.

Department of Anatomy, Cell Biology, Physiology and Biophysics (UNICAMP), Campinas, SP, Brazil.

Running Title: Heparanase–1 and apoptosis in the rat VP

Correspondence: Hernandes F. Carvalho

Department of Anatomy, Cell Biology, Physiology and Biophysics – Institute of Biology
State University of Campinas (UNICAMP)
13083–863 Campinas, SP, Brazil
Tel. 55 19 3521–6118
Fax: 55 19 3521–6185
e–mail: hern@unicamp.br

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ABSTRACT

Several enzymes are implicated in the remodeling of prostate tissue after androgen deprivation. We recently reported that the expression and activity of matrix metalloproteinases contribute to the occurrence of a second wave of cell death 11 days after castration (Int J Androl 33: 686, 2010). Here, we investigated whether heparanase-1, a heparan-sulfate degrading enzyme, could play a role in these events, using Western blotting, immunoperoxidase, *in situ* hybridization, and pharmacological inhibition experiments. The results demonstrate that heparanase-1 content and the amount of the active form (peak activation on day 10) increased after castration, that this enzyme shows a expression shift from the epithelium to the stroma (with contributions from both smooth muscle and fibroblasts), and that heparanase-1 inhibition with heparin, a competitive inhibitor, promoted a 52% reduction ($p<0.001$) in the number of apoptotic cells on day 11 after castration. Combined inhibition of heparanase-1 and MMPs (inhibited with GM6001, a broadly specific MMP inhibitor) was even more effective in inhibiting apoptosis. The results lead to the conclusion that extracellular matrix degradation induces anoikis and affects the rate of epithelial cell apoptosis after the first week of androgen deprivation.

Epithelial differentiation and the growth and physiology of the prostate gland depend on the expression of androgen receptors by some cell types and on paracrine and systemic regulation secondary to androgen stimulation. Epithelial growth and tumor invasion depend on the activity of several enzymes with the ability to degrade extracellular matrix (ECM) components and to release bound or cryptic bioactive fragments (Mott & Werb, 2004). Matrix metalloproteinases (MMP) are effectors of stromal loosening, allowing epithelial growth in the rat ventral prostate (VP) (Bruni-Cardoso & Carvalho, 2007; Bruni-Cardoso *et al*., 2008). Androgen ablation causes several regressive changes to the prostate, with a peak of epithelial-cell apoptosis 3 days after castration. Even after this peak of apoptosis, the ventral prostate continues to regress, reaching 10% of its original weight by day 21 after castration (Antonioli *et al*., 2007, Augusto *et al*., 2008). We have previously suggested (Garcia-Flórez *et al*., 2005) and recently confirmed that progressive regression after the first week of androgen deprivation requires stromal remodeling with the active participation of MMP-2, -7, and -9 (Bruni-Cardoso *et al*., 2010). Several enzymes are recruited to act in remodeling the prostate microenvironment during development and after castration. Recent studies demonstrated that MMPs are closely related to the second wave of epithelial cell death that occurs after castration, and are not responsive to the lack of androgen. MMP inhibition with doxycycline, hydrocortisone, or GM6001 decreased epithelial cell death by about 50%. This led us to suggest that additional factors besides MMPs contribute to inducing epithelial cell death (Bruni-Cardoso *et al*., 2010).

The ECM is a reservoir for a multitude of biomolecules with many functions. Heparan sulfate (HS) is involved in a variety of processes such as cell-cell interaction, cell adhesion,

proliferation, differentiation, morphogenesis, and structural integrity of the ECM, and binds both to structural proteins such as fibronectin and collagen, and to chemokines and growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) (Dempsey *et al*., 2000*;* Vlodavsky *et al*., 1996; Gospodarowicz *et al*., 1980*).* Heparanase-1 (Hpse-1) is a β-D-endoglucuronidase that cleaves HS chains, especially in low-sulfation sites, thereby releasing growth factors to the extracellular matrix (ECM). These factors activate several signaling pathways to coordinate cell fate and ECM remodeling. For example, HPSE-1 degrades the perlecan HS-chain and releases FGF10, which then modulates epithelial branching morphogenesis in the submandibular gland by increasing MAPK phosphorylation (Patel *et al*., 2007).

Under normal physiological conditions, Hpse-1 expression is restricted to activated leukocytes, endothelial cells, and smooth-muscle cells (Vlodavsky *et al*., 1992; Godder *et al*., 1991), as well as cytotrophoblasts, keratinocytes, and platelets (Dempsey *et al*., 2000; Vlodavsky *et al*., 1992; Goshen *et al*., 1996; Bernard *et al*., 2001). The expression of Hpse-1 is tightly controlled; however, it is commonly deregulated in tumor cells, which show increased heparanase activity (Koliopanos *et al*., 2001; Sato *et al*., 2004). A previous study demonstrated that Hpse-1, mRNA, and protein content increase after castration, correlating with a decreased amount of heparan sulfate and activation of stromal cells (Augusto *et al*., 2008).

The hypothesis of this study is that HPSE-1 is necessary for the induction of epithelial-cell death and contributes to the peak of apoptosis observed on day 11 after castration. To test it, we used immunohistochemistry and *in situ* hybridization to determine

variations in Hpse-1 content and to identify cell types expressing this enzyme, as well as the pharmacological inhibition of heparanase-1, to check their effects on epithelial cell death.

MATERIAL AND METHODS

ANIMALS

Fifty four three-month-old male Wistar rats were used. The rats were maintained in barrier facilities with free access to food and water, and were divided into sham-operated animals and animals assigned to 14 groups (three animals per group). Castration was achieved by bilateral orchiectomy through a scrotal incision under ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (10 mg/kg) anesthesia. Some animals in each group were killed on each day (days 1 through 14 after castration) by anesthetic overdose, and the ventral prostate was dissected, frozen in liquid nitrogen, and stored at -70ºC, or fixed according to the different protocols described below.

An additional 9 castrated animals were treated with GM6001 (0.7 mg/kg/day in PBS) (Sigma Chemical Co., St. Louis, MO, USA) on days 9 and 10; sodium heparin 5000 U/mL (571 U/kg/day in PBS) (Parinex, Hipolabor Pharmaceutics, Brazil) on days 8, 9, and 10; or GM6001 plus heparin, on days 8 to 10, in separate intraperitoneal injections. Three animals received only PBS, and were designated as the Cast11 group. All animals were killed on day 11, and had their VPs dissected and processed for paraffin embedding. Animal-handling and experimental procedures were approved by the State University of Campinas Committee for Ethics in Animal Experimentation (Protocol No. 1271-1).

PROTEIN EXTRACTION AND WESTERN BLOTTING

The tissue samples were weighed, homogenized in a Polytron homogenizer (Kinematica, Lucerne, Switzerland) for 1 min in 50 µL/mg lysis buffer containing 150 mM NaCl, 0.5% Triton X-100, 0.5 mg/mL EDTA, and 10 µL/mL protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 10,000 x*g* for 10 min. Protein concentration was determined using the Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and 50 µg protein was resolved in 12% SDS-PAGE gels under reducing conditions. After electrophoresis, proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, São Paulo, SP, Brazil). The membranes were blocked with TBS-T containing 1% nonfat milk and probed with goat anti-human heparanase antibody (HPA1, C-20) in TBS-T containing 0.1% nonfat milk (diluted 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with rabbit anti-goat IgG HRP-conjugated antibody (diluted 1:5000, Zymed-Invitrogen Laboratories, Carlsbad, CA, USA). Peroxidase activity was developed using an enhanced chemiluminescent substrate (Santa Cruz Biotechnology).

IMMUNOHISTOCHEMISTRY

Paraffin-embedded, paraformaldehyde-fixed tissues were cut into 4-µm sections, mounted on silane-treated slides, dewaxed in xylene, and rehydrated. The sections were briefly heated in a microwave oven in 10 mM citrate buffer, pH 6.0, and digested with 0.1% pepsin in 0.01 N HCl for 20 min at room temperature. The sections were blocked with 3% H_2O_2 for 10 min, followed by incubation with 1% of ABC kit serum (NCL-ABCu, Novocastra, Newcastle upon Tyne, England) for 1 h. The sections were incubated overnight with the same antibody against

heparanase (diluted 1:100) as described above. The tissue-bound primary antibody was detected with the ABC kit (NCL-ABCu, Novocastra). The sections were counterstained with methyl green.

IN SITU mRNA HYBRIDIZATION

For *Hpse-1* mRNA localization, all solutions and materials utilized were RNAse-free. The *Hpse-1* probe was determined and designed using the program Gene Runner 3.05 (Hastings Software, Inc.) The probe was synthesized and 5´-end labeled with biotin by Invitrogen Life Technologies (Carlsbad, CA, USA) as follows (anti-sense: (I) 5'- CAC TCT TGA CAT TAA CAC CTT GGG ACC TAC – 3', (II) 5'- GTA GAG AGT TAA ATC TCC TTC CCG ATA CCT T – 3', (III) 5'- CTT CAC TTA TTT GCC TCT TGG TCA TAT TGG – 3' and sense: (1) 5'- GTA GGT CCC AAG GTG TTA ATG TCA AGA GTG – 3', (II) 5'- AAG GTA TCG GGA AGG AGA TTT AAC TCT GTA C - 3' and (III) 5'- CCA ATA TGA CCA AGA GGC AAA TAA GTG AAG – 3', according to mRNA sequence (NM_022605) by NCBI.

Frozen sections were air-dried for 30 min at 37^{o}C, fixed using ice-cold 4% paraformaldehyde in PBS for 10 min, and washed twice in PBS for 5 min and twice in 2x SSC for 2 min. The sections were incubated with Proteinase K (20 μg/mL) for 10 min at room temperature and then washed twice for 5 min with 2x SSC. The sections were incubated in 0.1 M of tri-ethanolamine pH 8 (TEA Buffer) for 10 min and then with 0.25% acetic anhydride in TEA buffer for 10 min under magnetic stirring, and washed with 2x SSC. The pre-hybridization solution was composed of 50% formamide, 5x SSPE, Denhardt's solution (1x final concentration) completed with DEPC-treated water. The sections were pre-hybridized

for 3 h without the probe at 50°C in a humidified chamber with 50% formamide in SSPE. The probe mix was composed (for each tissue section) of 20 μL of pre-hybridization solution, 100 ng of riboprobe mix (anti-sense or sense), and 400 ng tRNA. The mixture was placed over the sections and incubated at 55°C overnight in a humidified chamber. After a 24-h hybridization step, the sections were washed four times with 4x SSC buffer for 10 and 5 min in PBS. The sections were incubated with a solution containing 0.3% Triton X-100, 3% BSA in PBS for 1 h at room temperature, and incubated with avidin-conjugated alkaline phosphatase (cat. no. 21321, Pierce, Rockford, IL, USA) diluted 1:250 for 3 h. After this procedure, the sections were washed three times for 10 min in PBS and incubated for 5 min in a solution containing 100 mM Tris-HCl, 100 mM NaCl, and 0.1% $MgCl_2$. The hybridization product was detected using alkaline-phosphatase substrate NBT/BCIP (Nitro Blue Tetrazolium/5-bromo-r-chloro-3-indolyl phosphate). The slides were counterstained with methyl green for 10 min, washed, and mounted in 90% glycerin in PBS.

STATISTICAL ANALYSIS

Statistical analyses were performed by ANOVA followed by the multi-comparison post-hoc Fisher's tests, at $p < 0.05$, using a free trial version of the software Minitab (Minitab Inc., State College, PA, USA). Results are presented as the mean ± standard error of the mean of measurements for at least three animals per group.

RESULTS

HEPARANASE CONTENT WAS INCREASED AFTER CASTRATION AND SHOWED A PEAK OF ACTIVATION AT DAY 10

Western blotting revealed two bands of Mr= 65 kDa and 50 kDa, corresponding to the heparanase-1 precursor pro-enzyme and active form, respectively (Fig. 1A). Measurements of band intensity by densitometry showed an increase in heparanase-1 (pro-enzyme plus active form) content, starting 3 days after castration. As a measure of the heparanase-1 activation level, we used the ratio between the active band and the total amount of heparanase-1, and this revealed a predominance of the active form between days 7 and 10 after castration (Fig. 1B).

HEPARANASE-1 SHIFT FROM THE EPITHELIUM TO THE STROMA AFTER CASTRATION

The tissue distribution of heparanase-1 was determined by immunohistochemistry. The enzyme was located at the epithelial cell surface, with increased intensity at the tips of the epithelial infolds (Fig. 2A, inset). Some staining was also seen in the stroma compartment, particularly associated with the surface of smooth-muscle cells. Staining of blood vessels was related to the presence of platelets and endothelial cells. Castration resulted in increased immunostaining of the stroma in which would be related to increased vascular permeability and increased content of platelets on day 3 after surgery (Fig. 2B, inset). On day 14 after castration, immunostaining was abolished in the epithelium and was concentrated in the smooth-muscle cells around the epithelium (Fig. 2C).

To distinguish between local production of the enzyme and a probable artifactual increase in the content of heparanase-1 between days 3 and 7 (Fig. 1) due to increased platelet extravasation, and to identify cells expressing Hpse-1 in the rat VP, we used *in situ* hybridization to locate cells expressing Hpse-1 mRNA in the VP and their changes after castration. Figure 2D shows the result obtained for the VP from a non-castrated animal, demonstrating a predominance of reaction in the epithelium and little contribution by the stromal cells (Fig. 2D, inset). Seven days after castration (Fig. 2E), the tissue changes were visible and associated with concentrated staining in the epithelial cells but not with the diffuse pattern seen before castration, whereas a clear increase in the reaction was observed in the stromal cells. Fourteen days after castration, the staining of the epithelium was much reduced and restricted to individual cells recognized as macrophages (Fig. 2F).

HEPARANASE-1 COMBINES WITH MMPS TO INDUCE EPITHELIAL-CELL DEATH AFTER CASTRATION

The expression and activation pattern of heparanase-1 in the VP in response to castration was similar to those of MMP-2, -7, and 9 (Bruni-Cardoso *et al*., 2010). We then asked whether heparanase-1 activity combined with MMPs in causing epithelial-cell death. We employed heparin, a competitive substrate for heparanase alone or in combination with GM6001, a broadly specific MMP inhibitor, and determined its effect on the epithelial apoptotic index after TUNEL-labeling. Figure 3 shows that heparin alone caused a significant decrease in the percentage of TUNEL-positive epithelial cells, although this decrease was less significant than the drop caused by GM6001 alone. Simultaneous administration of heparin

and GM6001 enhanced the inhibition of epithelial-cell death, as revealed by the highly significant smaller number of dying cells with respect to the untreated group.

DISCUSSION

In the present study, we extended our analysis of the expression of heparanase-1 in the rat ventral prostate, by showing the variation in content and percentage of the active form, which reaches a peak on day 10 after castration. We also demonstrated a shift from epithelial to stromal expression, and identified both smooth-muscle cells and fibroblasts as stromal cells expressing heparanase-1. In addition, using heparin, a competitive inhibitor of heparanase-1, we demonstrated that inhibition of heparanase-1 promoted a 52% decrease the number of apoptotic cells on day 11 after castration.

We have previously reported that the rat VP showed enhanced expression of heparanase-1 after castration (Augusto *et al*., 2008), and that this increase was associated with specific aspects of epithelial- and stromal-cell interactions to cause changes in the basement membrane (Carvalho & Line, 1996). We have also previously demonstrated that altering the rate of epithelial cells did not affect the ratio of epithelial regression up to one week after castration (Garcia-Florez *et al*., 2005), which led us to suppose that epithelial regression depended on stromal remodeling. To check this hypothesis, we investigated the pattern of expression of MMPs in the rat VP on a daily basis, and demonstrated not only that MMP-2, -7, and -9 expression and activation increased after castration, but also that this enhanced MMP activity preceded a previously unknown peak of apoptosis that occurs on day 11 after castration. We also showed that inhibiting MMP activity with different pharmacological drugs reduced the proportion of apoptotic-cell death to about 50%. This limited reduction led us to

suggest that additional factors contribute to inducing epithelial-cell apoptosis (Bruni-Cardoso *et al*., 2010). It is interesting to note that the peak of MMP activation that we observed coincides with an earlier report of a peak of plasminogen activators 10 days after castration (Rennie et al., 1984) and that their inhibition with cortisol suppressed cell deletion in a dose-dependent fashion (Rennie et al., 1988).

The experiments reported here demonstrated that heparanase-1 content increases after castration, and reaches a peak of activation 10 days after surgery. This suggests a temporal relationship between the two events. To demonstrate a direct causal relationship between them, we used heparin as a competitive inhibitor of heparanase-1. The results demonstrated that heparin was capable of reducing the number of apoptotic cells on day 11. To further explore this phenomenon, we have tested the combined effect of heparin and GM6001 and demonstrated a even greater reduction (68%) in the mean number of apoptotic cells in comparison with the untreated animals. Besides confirming our hypothesis that heparanase-1 is necessary for the epithelial-cell apoptosis, we propose a functional synergy between heparin and GM6001 and the absence of dualism (Ariëns et al., 1964) in reducing the percentage of apoptotic cells on day 11 (Fig. 4), which remains to be explored in deep in an mimetic system that does not involve an unsustainable number of experimental animals.

As an analog of the natural substrate of heparanase, heparin is commonly considered a potent inhibitor of heparanase. This activity is attributed, in part, to its high affinity to the enzyme and limited degradation, serving as an alternative substrate (Vlodavsky et al., 1994; Nakajima et al., 1988). Studies have developed heparin-based anticancer lead compounds in order to inhibit heparanase action without causing side effects (Naggi *et al*., 2005).

Based on the present and previous results, we believe that the apoptosis peak on day 11 after castration is modulated by, but is not exclusively dependent on uPA (Rennie et al., 1988), MMPs (Bruni-Cardoso et al., 2010), and Hpse-1. This suggests that matrix-degrading enzymes contribute to inducing anoikis, which modulates the rate of cell death in this period after castration. We cannot rule out the possibility that enzyme activity on the extracellular matrix could release factors inducing apoptosis of the epithelial cells, as suggested for the action of MMP-7 in cleaving FAS-L in the mouse prostate (Powell et al., 1999).

Our current view of the epithelial regression in response to castration is that after a first peak of apoptosis due to androgen deprivation, further regression will depend on additional mechanisms taking place at specific time points. We have shown that stromal remodeling is a key factor in this scenario, and contributes to inducing epithelial-cell death by causing anoikis in the epithelial cells. We believe that castration-induced changes in the prostate gland are poorly explored and significantly affect the prognosis and recurrence in the androgen-independent form of prostate cancer in humans. Knowledge from experimental animals is then important for the understanding of molecular mechanisms regulating these changes to modulate the prostate environment and perhaps interfere with appropriate measures aiming at better outcome and later recurrence

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Legends of the figures

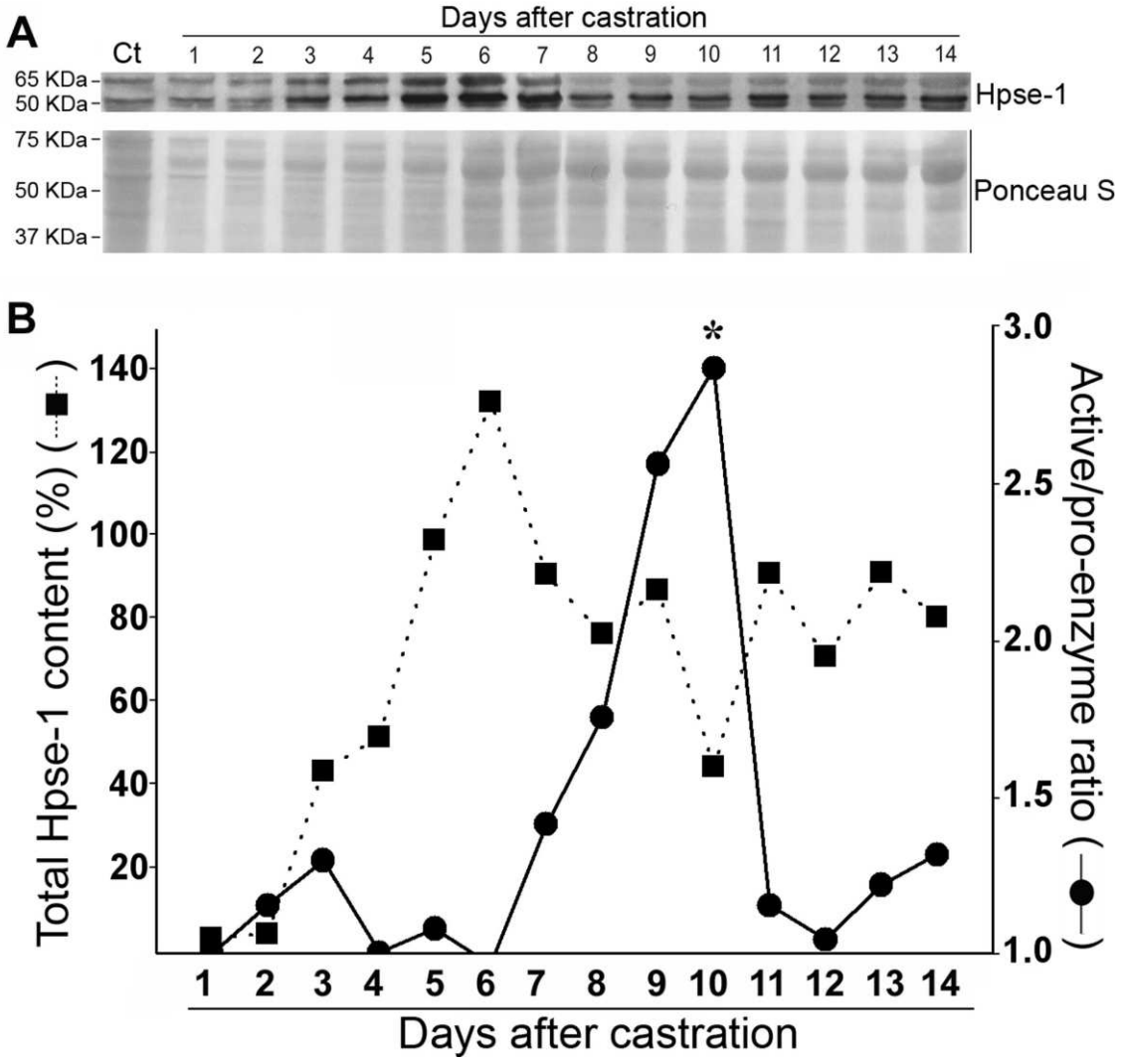
Figure 1 – Hpse-1 content variation after castration. (A) A representative Western blotting using an anti-Hpse-1 antibody, in which the pro-enzyme (Mr= 65 kDa) and the active form (Mr = 50 kDa) were identified in the VP of non-castrated (Ct) and castrated rats (days 1-14 after surgery). GAPDH was used as loading control (Mr = 37kDa). (B) After densitometry, the relative variation in the total amount of the enzyme (pro-enzyme and active form) (squares) increased three days after castration, while the ratio between the active form and the pro-enzyme (circles) showed a clear peak with a significant increase 10 days after castration. The results are the means of four measurements for four animals at each time point. The error bars were omitted for the sake of clarity. The asterisk indicates a significant increase in the ratio between the active form and the pro-enzyme after ANOVA followed by Fisher's test ($p<0.05$).

Figure 2 – Immunoperoxidase (A-C) and *in situ* hybridization (D-F) localization of Hpse-1 protein and mRNA, respectively, in the rat VP before (A and D) and after castration (B-C and E-F). Hpse-1 was found in the rat VP both in the epithelium (Ep) and stroma (A). In the epithelial cells, the staining was concentrated at the basolateral cell surface and in the tips of the epithelial infolds (inset). (B) Castration resulted in a progressive increase in the amount of Hpse-1 in the stroma (7 days after surgery) and sites of platelet extravasation (B, inset; 3 days after surgery). (C) Fourteen days after castration, the Hpse was concentrated in the stroma. (D) ISH showed the location of the Hpse-1 mRNA in the epithelium and in the stroma. The inset shows individualized fibroblasts in the stroma with a positive reaction for Hpse-1 mRNA. (E) Seven days after castration, the signs of epithelial atrophy are evident and the

staining in the epithelium is less abundant, while the staining in the smooth-muscle cells is more prominent. (F) Fourteen days after castration, the staining in the epithelium was restricted to a few individualized cells, recognized as macrophages, while the staining of smooth-muscle cells and fibroblasts in the stroma was evident. Figs. A-F, and inset to Fig. B, bars = 50 μm; insets to Fig. A and D, bars = 10 μm.

Figure 3 – Percentage of TUNEL-positive epithelial cells on day 11 after castration (Cast11) and after treatment with heparin, GM6001, or a combination of both. The percentage of apoptotic cells was reduced after treatment with either heparin or GM6001. A highly significant reduction in apoptosis rate (ANOVA, followed by Fisher's test; $p < 0.000001$) was found when a combination of the two inhibitors was used. Results are the mean ± standard error of the mean (n=3 animals per experimental group; 12 microscope fields per animal).

Figure 1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Total Hpse-1 content (%) (---■---)
Active/pro-enzyme ratio (—●—)
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Days after castration

Figure 2

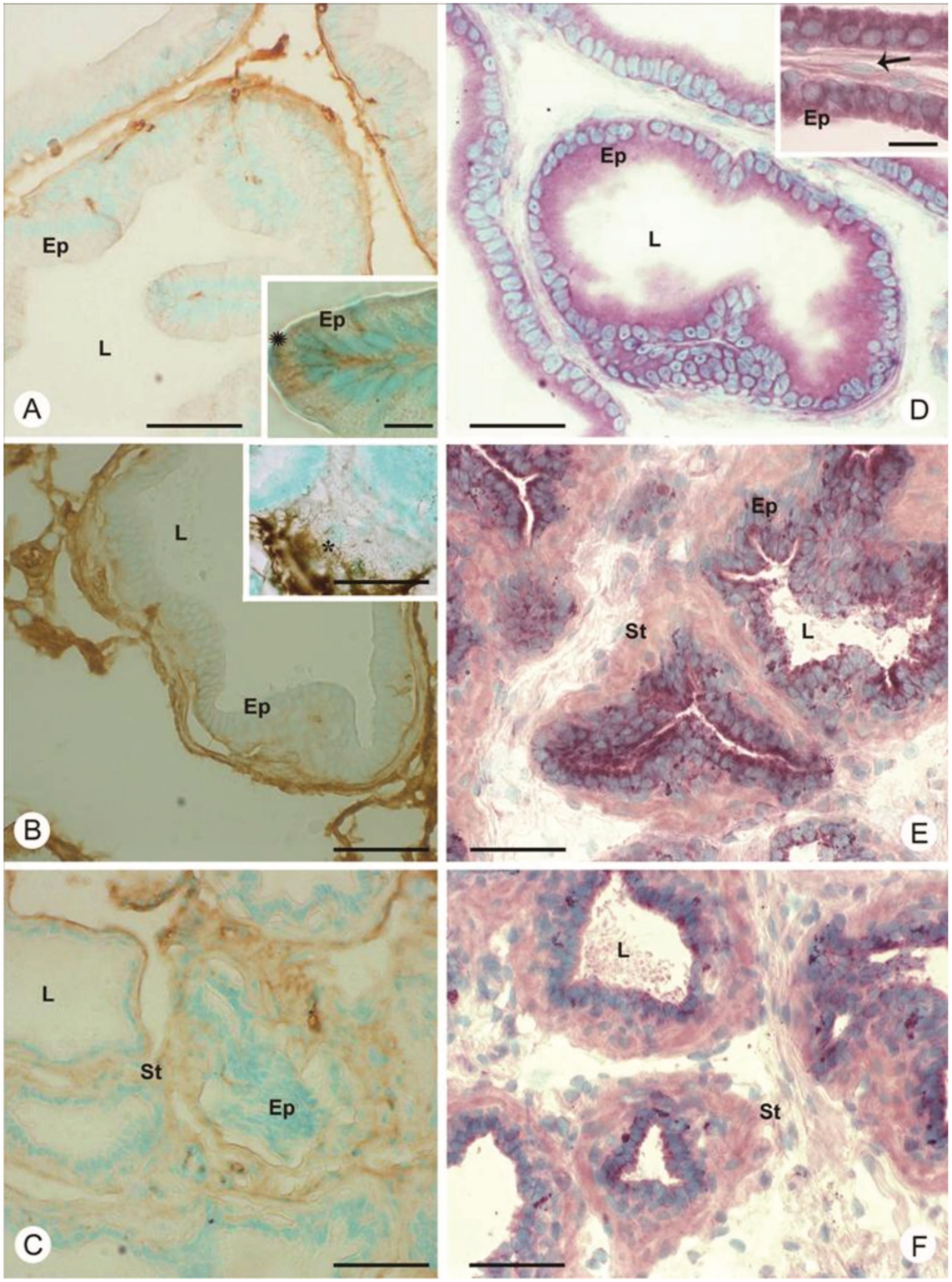
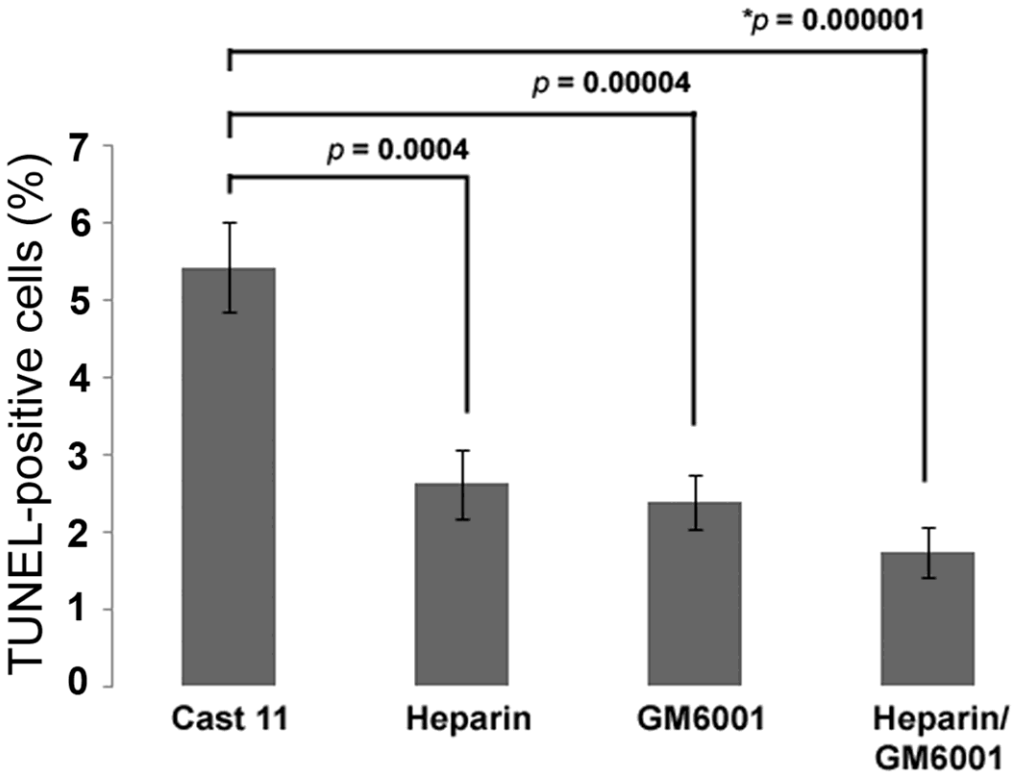


Figure 3


*p = 0.000001
p = 0.00004
p = 0.0004
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4.2 MANUSCRITO II : Publicado no *International Journal of Andrology* (2010)

"*Oestrogen imprinting causes nuclear changes in epithelial cells and overall inhibition of gene transcription and protein synthesis in rat ventral prostate*"

Autores: Taize M. Augusto, Alexandre Bruni–Cardoso, Danilo M. Damas–Souza, Willian F. Zambuzzi, Fabiana Kühne, Luciana B. Lourenço, Carmen V. Ferreira and Hernandes F. Carvalho.

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ORIGINAL ARTICLE

Oestrogen imprinting causes nuclear changes in epithelial cells and overall inhibition of gene transcription and protein synthesis in rat ventral prostate

T. M. Augusto*, A. Bruni-Cardoso*, D. M. Damas-Souza*, W. F. Zambuzzi†, F. Kühne*, L. B. Lourenço*, C. V. Ferreira† and H. F. Carvalho*

*Department of Anatomy, Cell Biology, Physiology and Biophysics, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil, and †Department of Biochemistry, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil

Keywords:
4EBP, androgen receptor, oestrogen imprinting, mTOR, nucleolus, prostate, protein synthesis

Correspondence:
Hernandes F Carvalho, Department of Anatomy, Cell Biology, Physiology, and Biophysics, Institute of Biology, State University of Campinas (Unicamp), CP6109, 13083-863 Campinas, SP, Brazil.
E-mail: hern@unicamp.br

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Summary

Oestrogen exposure during the early post-natal period affects male growth, physiology, and susceptibility to disease in adult life. The prostate gland is susceptible to this oestrogen imprinting, showing a reduced expression of the androgen receptor and inability to respond to androgen stimulus. In this context, we decided to study key signalling regulators of ventral prostate (VP) functioning after early postnatal exposure to high-dose oestrogen. Our results showed a decrease of mTOR phosphorylation and its direct downstream target 4EBP. It is known that mTOR-induced signalling is a pivotal pathway of cell metabolism, which is able to control gene transcription and protein synthesis. We then decided to investigate other indicators of a reduced metabolism in the oestrogenized prostate, and found that the luminal epithelial cells were shorter, less polarized and had smaller nuclei containing more compacted chromatin, suggesting that a general mechanism of regulating gene expression and protein synthesis could be installed in the epithelium of the oestrogenized VP. To evaluate this idea, we analysed nucleolar morphology, and measured the amount of ribosomes and the level of methylation of the 45S ribosomal RNA promoter region. These data indicated that the nucleolus was dismantled and that the methylation at the 45S promoter was increased (~five-fold). Taken together, the results support the idea that the oestrogenized prostate maintains a very low transcriptional level and protein turnover by affecting canonical signalling pathways and promoting nuclear and nucleolar changes.

Introduction

Prostate differentiation, growth and functionality depend on androgen signalling. Testosterone and dihydrotestosterone exert their functions through the androgen receptor (AR), which regulates the expression of several genes related to male development, physiology and behaviour. In general, oestrogens have an anti-androgenic effect by inhibiting the hypothalamic-pituitary-testicular axis and then reducing the production of testosterone by Leydig cells. Moreover, oestrogens are able to increase the production of prolactin, thereby affecting the prostate gland (Gilleran *et al.*, 2003). In addition, local expression of oestrogen receptors, as well as their signalling cascades, indicate direct effects on the prostate physiology (Jarred *et al.*, 2000, 2002; Prins *et al.*, 2001; Risbridger *et al.*, 2001; Bianco *et al.*, 2002; Garcia-Florez *et al.*, 2005; Prins & Korach, 2008).

It has long been known that foetal exposure to maternal oestrogen causes epithelial squamous metaplasia of the human prostate, and that this is reversed soon after birth as oestrogen levels decrease (Driscoll & Taylor, 1980; Yonemura *et al.*, 1995). Furthermore, exposure to 17β-oestradiol (E2) also results in persistent distortion of the ductal structure (Yonemura *et al.*, 1995), and neonatal exposure to E2 or other oestrogenic molecules, such as

diethylstilbestrol, results in prostate imprinting. The prostate gland undergoes a series of developmental changes during the first three postnatal weeks and different developmental settings take place during the time of oestrogen administration (Hayashi *et al.*, 1991; Prins *et al.*, 2006; Bruni-Cardoso & Carvalho, 2007).

A detailed study of this physiological phenomenon confirmed a non-normotonic response, meaning that low doses promote growth whereas high doses result in impaired growth, in a typical inverted-U-shaped curve (Naslund & Coffey, 1986; Santti *et al.*, 1994; Putz *et al.*, 2001). Furthermore, exposure to oestrogen during early postnatal life affects the hypothalamic imprint, with effects on brain defeminization and masculinization (Gerardin *et al.*, 2008).

It was later shown that AR is actively degraded in a proteasome-dependent way (at postnatal day 10) after oestrogen exposure on postnatal days 1, 3 and 5 (Woodham *et al.*, 2003), eventually resulting in the lack of AR immunostaining in the prostate gland (Putz *et al.*, 2001). This, in turn, is associated with reduced response to androgens during puberty, which seems to be a major event responsible for impaired prostate growth and development. In this respect, oestrogen imprinting deserves attention, because it might interfere with male phenotype acquisition and psychosocial behaviour.

Recently, it has been demonstrated that some genes are epigenetically regulated after early post-natal exposure to oestradiol or bisphenol A. Among these genes was the gene coding for phospho-diesterase type 4, which was shown to undergo methylation-dependent expression modulation (Ho *et al.*, 2006).

In this study, we investigated aspects of the rat ventral prostate that could be related to impaired physiology of the gland. The profile of mTOR (mammalian target of rapamycin) cascade was shown to be down-regulated in the gland; and then, histological changes, tissue compartment organization, nuclear and nucleolar morphometrics, the amount of rRNA and the methylation status of the ribosomal RNA precursor 45S gene promoter were assessed in the epithelial cells. Taken together, our results demonstrated the existence of an overall inhibition of transcription and protein synthesis in the epithelial cells of the ventral prostate of oestrogenized rats.

Material and methods

Materials

Anti-4EBP (eukaryotic translation initiation factor 4E binding protein) (cat. 2845), anti-phospho-4EBP (cat. 2855), anti-mTOR (cat. 2983) anti-phospho-mTOR (cat. 2971) (all diluted 1 : 1000) and peroxidase-conjugated horse anti-mouse IgG (cat. 7076) (diluted 1 : 2500) were obtained from Cell Signalling (Beverly, MA, USA). Peroxidase-conjugated goat anti-rabbit IgGs (cat. 81-6120) (diluted 1 : 2500) were obtained from Zymed (San Francisco, CA, USA). All chemicals were of analytical grade.

Animals, treatments and tissue processing

Male Wistar rats received 120 μg 17β-oestradiol (E2) in 25 μL corn oil (corresponding to 15 mg/kg body weight; mean animal body weight 8 g) at postnatal days 1, 3 and 5, to achieve most of the effects compromising ventral prostate growth, as described by Putz *et al.* (2001). These animals were kept under normal light conditions and received filtered tap water and Purina rodent chow ad libitum until postnatal day-90, when they were killed by anaesthetic overdose after xylazin and ketamin anaesthesia. The animal-handling and experimental protocols were approved by the University's Committee for Ethics in Animal Experimentation (Protocol no. 1248–1).

Animals were weighed and their ventral prostate and testes dissected out, weighed and either frozen in liquid nitrogen or fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h. Fixed prostates were then processed for paraffin or historesin embedding.

Protein extraction and blotting

Rat ventral prostate samples were fragmented with a scalpel blade and washed three times with cold PBS, before being homogenized using a Polytron (Kinematica, Lucerne, Switzerland) in lysis buffer [0.32 mM sucrose, 10 mg/mL leupeptin, 10 mg/mL soybean trypsin inhibitor, 2 mg/mL aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM n,4 dithiothreitol (DTT), 10 mM reduced glutathione, 5% SDS in 50 mM Tris-HCl pH 7.4], incubated in an ice bath for 2 h. After this period, the material was cleared by centrifugation at 29 000 ***g*** for 15 min and the supernatant recovered for protein determination using Bradford's microassay (Bio-Rad Laboratories, Hercules, CA, USA). One hundred μg protein was resolved in SDS-PAGE under reducing conditions before electro-transferring to nitrocellulose membranes (Amersham Biosciences, São Paulo, SP, Brazil) for 1 h at 400 mA. For mTOR, we used a slot blot procedure, using 75 μg protein, because we encountered problems in resolving this protein in SDS-gels and transferring it to nitrocellulose membranes. The transferred membranes were washed with Tris-buffered saline (TBS) and incubated with 5% skim milk powder in TBS containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. After blocking, the membranes were incubated with a specific primary antibody solution containing 5% BSA (bovine serum albumen) in TBS-Tween overnight at 4 °C. After

washing with TBS-Tween, the membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibodies and the reaction developed using the chemoluminescent reagent Luminol (Santa Cruz Biotech, Santa Cruz, CA, USA). The intensity of the bands was determined by densitometry using the Scion Image Software (Scion Corporation, Frederick, MD, USA).

Histology and stereology

Historesin sections (2 μm) were stained with haematoxylin and eosin for general histology and stereology according to Huttunen *et al.* (1981), as described previously (Garcia-Florez *et al.*, 2005; Antonioli *et al.*, 2007). Twelve microscope fields/oestrogenized animal ($n = 3$) and six microscope fields/control animal ($n = 5$) were analysed.

Nuclear morphometrics

Prostate sections (5 μm) were treated with 100 mM sodium borohydride for 5 min and then submitted to the Feulgen reaction. Following hydrolysis in 4N HCl at room temperature for 1 h and 15 min, sections were incubated with Schiff's reagent for 45 min at room temperature in the dark. Histomorphometry was applied to epithelial cell nuclei that were not superimposed on each other, using the Image Tool freeware. The parameters analysed were nuclear area and perimeter, integration of optical densities, forma factor and standard deviation of optical densities.

Silver impregnation and nucleolar morphometry

Nucleolar morphometrics were performed after silver impregnation. In detail, prostate sections (5 μm) were dewaxed and rehydrated before incubation with a 2 : 1 (v : v) solution prepared with 50% silver nitrate and 2% gelatin (Synth, Diadema, SP, Brazil) in 1% formic acid at 60 °C for 7 min, covered with a glass cover slip. After incubation, the cover slips were removed and the sections were washed with distilled water, dehydrated, mounted in glycerol and documented immediately. Cell nuclei that were not superimposed on each other were isolated from the images for the construction of a nuclei gallery, avoiding nuclear calottes. The images were then segmented manually using the Image J freeware. The parameters analysed were the number of silver-reactive dots per nucleus, the total area and the ratio between the total area of the silver-reactive material and nuclear area. Data are presented as the distribution of silver-reactive dots per nucleus and the correlation between the number of particles and the total area of the silver-reactive material, and the correlation between the number of dots and the nuclear area fraction occupied by silver-reactive material. We followed the recommendations by González-Pardo *et al.* (1994) to avoid bias in the quantification.

Acridine orange staining for the simultaneous identification of DNA and RNA

Paraffin sections of VP from the control and oestronized rats were stained with diluted solution of acridine orange followed by counterstaining with fast green at pH 2.5 for quenching non-specific acridine orange staining of proteins (protocol modified by Damas and Carvalho, unpublished data). The stained material was then observed under the fluorescence microscope. Controls were carried out by acid hydrolysis (4N HCl for 45 min) or RNase A 20 μg/mL for 30 min at room temperature.

Percoll isolation of prostate epithelial cells and DNA extraction

Epithelial cells from the ventral prostate were isolated using Percoll (Sigma Chemical Co., Saint Louis, MO, USA) gradient centrifugation as described by Liu *et al.* (1997). The resulting preparations contained more than 95% epithelial cells, as judged by subsequent culturing (not shown). The isolated cells were resuspended in RPMI and counted in a Neubauer chamber. Five-to-ten-million-cell aliquots were prepared, and DNA was isolated using Trizol reagent (Invitrogen, São Paulo, SP, Brazil), according to the manufacturer's instructions. DNA content and purity were assessed by spectrophotometry, using an Ultraspec 2100-Pro spectrophotometer (Amersham Biosciences). DNA (2.2 or 22 ng) was digested in 40 μl reaction volume, following the instructions of the manufacturer, except that the incubation time was extended to 1 h. The enzymes employed were FastDigest Hpa II (Cat. FD0514; Fermentas, Glen Burnie, MD, USA) and FastDigest MspI (Cat. FD0544; Fermentas). Non-digested DNA was used as the control. The reaction product was submitted to qPCR, with no further purification. The $\Delta\Delta C_T$ method was used for comparison of the reaction products.

Analysis of the methylation status in the 45S rDNA promoter

To estimate the level of methylation of the 45S promoter region in epithelial cells, we applied a protocol based on the existence of a specific CCGG site with respect to the transcription initiation site, which is conserved from plants to mammals (Sardana *et al.*, 1993; Stancheva *et al.*, 1997), including rats, as reported by McStay & Grummt (2008). Restriction enzymes *Msp*I and *Hpa*II recognize and digest the CCGG sequence, but *Hpa*II is only active if the site is non-methylated (Singer *et al.*, 1979). For

quantification, we designed the pair of primers P1 (45S, 163 bp) (50 nM) 5′-ACCAGTTGTTCCTTTGCGGTC-3′ and P2 (45S, 59 bp) 5′-ATAAACATGACAGGCCACAC GG-3′, flanking the CCGG motif, based on the GenBank sequence for the 45S rDNA (X00677), so that cleavage of the site would impair PCR amplification (Fig. 1). The comparison between the amplification of non-digested DNA (22 ng) and *Hpa*II-digested DNA (1 μL enzyme/5 μg DNA; 1 h incubation; Fermentas) is then an estimate of the methylation level at this specific site. DNA (22 ng) digestion with *Msp*I (1 μL enzyme/5 μg DNA; 1 h incubation; Fermentas) was used to determine the basal level of amplification, as a result of possible polymorphisms hindering enzyme cleavage but still allowing primer pairing. The reaction product (2.2 ng of digested DNA) was quantified by qPCR using 25 μL SyBR Green two-step singleplex reaction, using the single copy gene β-actin as an internal control (NM_031144.20) and the primers (50 nM) Forward-5′-CTGGCCTCACTGTC-CACCTT-3′ and Reverse-5′-GGGCCGGACTCATCG-TACT-3′).

Statistical analysis

Differences were determined by the two-tailed *t*-test at $p < 0.05$, using a free trial version of the software Minitab.

Results

Body, testes and ventral prostate weight

The biological model explored in this study caused no difference in body weight when compared with the controls at postnatal day 90 [Fig. 2(a)] by using high doses of E2 in rats in early postnatal life. On the other hand, testis and prostate-gland growth was affected by the treatment, which significantly reduced the sizes of both organs [Fig. 2(b–d)], reproducing the usual effects of neonatal high-dose E2-exposure.

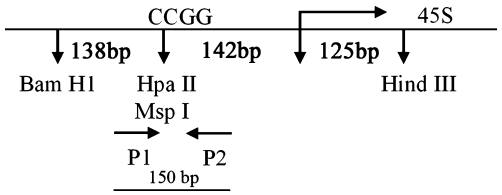

Figure 1 Schematic drawing of the strategy to determine the level of methylation of a single CpG in the 45S gene promoter. The percentage methylation of a CCpGG site was investigated, by comparing the cleavage by the restriction enzyme Msp I (methylation insensitive) or Hpa II, which only cleaves the non-methylated sequence. Cleavage sites of BAM H1 and Hind III are also shown (Sardana *et al.*, 1993; adjusted according to available sequence for the rat, AC_00081; 35560300 bp to 35560768 bp), as well as the position of the primer set employed for PCR amplification of a 104 bp amplicon (P1, sense and P2, reverse).

mTOR signalling was compromised in prostate from oestrogenized animals

Following the demonstration of an association between the energy status of the cell and the synthesis of ribosomes (Mayer *et al.*, 2004; Murayama *et al.*, 2008), and in an attempt to identify the possible mechanism involved in the metabolic modulation supposed to occur in the oestrogenized prostate, especially concerning protein synthesis, we investigated whether the mTOR-induced signalling could be involved. It is widely accepted that mTOR is a Ser/Thr protein kinase that functions as an ATP- and amino-acid sensor to balance nutrient availability and cell growth (Mayer *et al.*, 2004). When sufficient nutrients are available, mTOR responds to a phosphatidic acid-mediated signal to transmit a positive signal to p70 S6 kinase and participate in the inactivation of the eIF4E inhibitor, 4E-BP, translation repressor protein (also known as PHAS-1) (Pause *et al.*, 1994; Wullschleger *et al.*, 2006). Data from slot blotting showed reduced (~53%) phos-

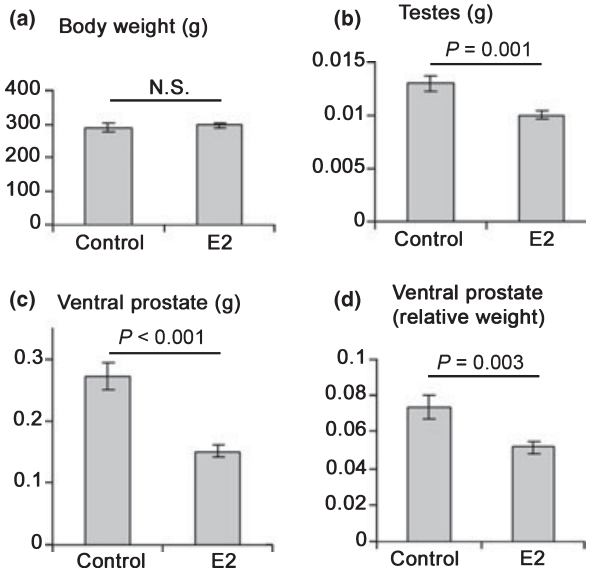

Figure 2 Evaluation of the effect of neonatal oestrogenization in body (a), testes (b) and ventral prostate weight (c) at 90 days of age. There was no effect on body weight, whereas the testes and ventral prostate showed significantly reduced weights. The relative weight (d) (ratio between the gland and body weight) of the ventral prostate was similarly reduced, showing reproducibility of the oestrogenization treatment.

phorylation of mTOR in oestrogenized rats compared with the controls [Fig. 3(a, b)].

More interestingly, data from western blotting also showed a larger amount (83% increase) of 4E-BP protein [Fig. 3(c)], associated with a 63% reduction in its phosphorylated form [Fig. 3(d)].

Histological changes and variations in tissue compartments

Histological analysis of the prostate gland showed remarkable changes in the epithelium. Cells in the intermediate region of the ductal system appeared smaller, shorter, vacuolated and had smaller nuclei containing condensed chromatin, when compared with the tall cylindrical cells observed in the controls. The nucleolus was also not clearly seen in the oestrogenized organ (Fig. 4). Early postnatal high-dose oestrogen also resulted in atrophic smooth muscle cells (Fig. 4). Data from stereology revealed a reduction in the volume density of the epithelium and smooth muscle cells, which were counterbalanced by increased volume density of the lumen and non-muscular stroma [Fig. 5(a)]. Analysis of the volume occupied by each compartment showed significant reduction in the epithelium, lumen and smooth muscle cells, but not in the stroma, with the most striking modification being a 66% reduction in the epithelial volume [Fig. 5(b)].

Nuclear changes

Morphometric analysis of the epithelial cell nuclei demonstrated that those in oestrogenized animals exhibited a smaller nuclear area, perimeter and integration of the optical densities, were less rounded (smaller forma factor), and had more-uniformly compacted chromatin (smaller standard deviation) than the nuclei in the ventral prostate of controls (Table 1).

Nucleolar changes

Next, we extended the analysis of the cell nucleus and searched for a general mechanism of regulating VP epithelial cells in oestrogenized animals. Therefore, we investigated whether the observed changes (Fig. 6) in the nucleolus could be measured and represent actual changes in nucleolar structure and function in the epithelial cells of oestrogenized animals. The first approach was to measure morphometric parameters after silver impregnation [Fig. 6(a, b)]. By counting the number of silver-reactive dots per cell nucleus, we revealed an increase of the number of dots in oestrogenized animals. The mean number of dots in the control animals was 4 ± 1.9, and increased to 6 ± 2.9 ($p < 0.001$) in oestrogenized animals. It was easy to find 10 or more dots in the oestrogenized animals, whereas the highest number of dots found in the controls

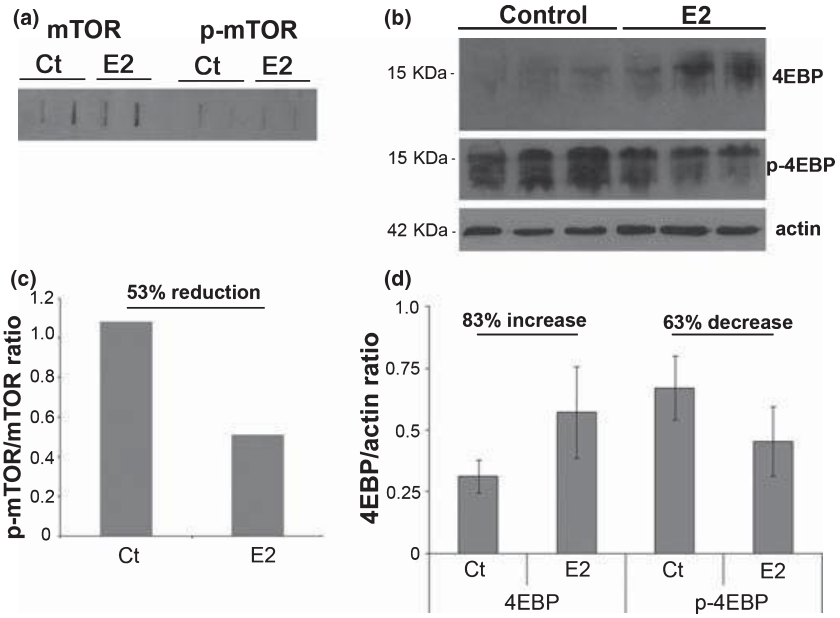

Figure 3 Slot blotting for mTOR and its phosphorylation form (a) in control and oestrogenized animals. (b) Representative Western blotting for 4EBP and its phosphorylation form. (c) Densitometric quantification of the results for mTOR, showing the reduced ratio between the phosphorylated and total mTOR ($n = 2$). (d) Densitometric quantification of the results, evidencing an 83% increase in protein and a 63% reduction of the phosphorylated form, after normalization using total actin ($n = 3$).

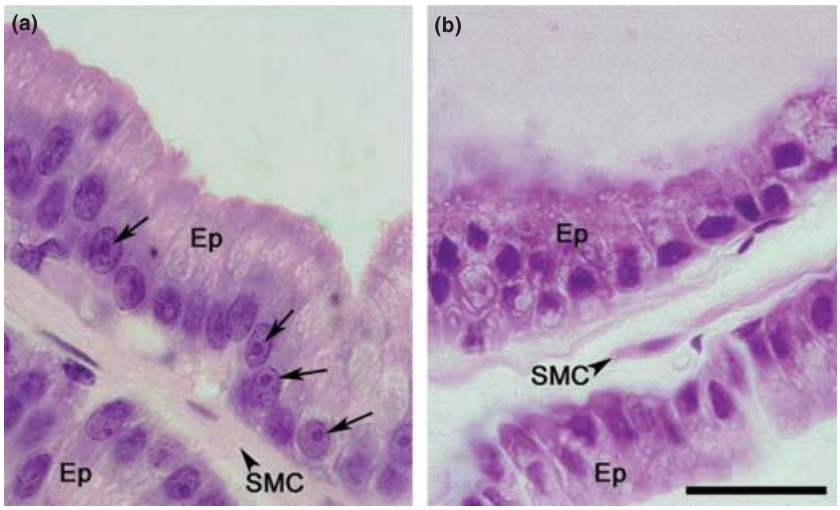

Figure 4 Histology of the rat ventral prostate in control (a) and oestrogenized rats at postnatal day 90 (b). There was a striking modification of the epithelium (Ep), with short and vacuolated luminal cells. Their nuclei have an irregular outline and condensed chromatin. The nucleoli are not easily evidenced, as in the control (arrows). Likewise, the supranuclear hypochromic region, which is a hallmark of epithelial polarization, is lost in oestrogenized rats. Smooth muscle cells (SMC) are atrophic in oestrogenized rats. Bar = 50 μm.

was 9 [Fig. 6(c)]. Furthermore, an increased number of silver-impregnated dots in the controls were positively correlated with the nucleolar area, whereas this was not found in the oestrogenized animals [Fig. 6(d)]. This aspect was even more evident when the number of silver-reactive dots was correlated with the nuclear fraction occupied by the nucleolus (or nucleolus-associated material) [Fig. 6(e)]. The linear regression of the data for the oestrogenized animals showed a slightly negative slope, indicating that the nucleolus occupies a slightly smaller fraction of the nuclear volume as it is dissociated into a larger number of dots. This demonstrates that, in the controls, the dissociation of individual nucleolus organizer regions (NORs) correlates with an increased amount of nucleolus-associated material (RNA and protein) and hence with greater nucleolar activity. By contrast, in the oestrogenized rats, dissociated dots represent NORs with no association of additional nucleolar components (i.e. RNA and proteins) that contribute to the nucleolar mass (and consequently to its nuclear area fraction). This strongly suggests a reduced nucleolar activity and compromised ribosome biogenesis.

RNA content in epithelial cells

Considering the apparent reduction in transcription, as indicated by the compacted nuclei and reduced nucleolar activity, we decided to adapt a histochemical procedure for the simultaneous identification of DNA and RNA based on acridine-orange staining and fast-green counterstaining (to reduce non-specific staining of proteins). Figure 7 shows the presence of a relatively large amount of RNA in the epithelial cells. In oestrogenized animals, the VP epithelium contained a smaller amount of RNA, mostly located in the supranuclear region, and likely representing residual ribosomes.

Methylation status of the 45S rDNA promoter

To check whether the nucleolar changes were related to the methylation status of the 45S rDNA promoter, we investigated the level of methylation of a single CCGG site in the ribosome precursor 45S promoter region at position – 145 by using restriction enzymes and PCR amplification strategies. Genomic DNA was digested with *Hpa*II (McStay & Grummt, 2008), which does not digest methylated sites, and Hsp1, which is methylation-insensitive, as detailed in Material and methods. Reaction products were then amplified by *q*PCR and compared with non-digested DNA. Figure 8 demonstrates that whereas in the control VP the intensity of *Hpa*II was about the same as that of *Msp*I (i.e. 14% and 19% of the control non-digested DNA respectively), the digestion of the DNA from the oestrogenized VP was almost completely insensitive to *Hpa*II, i.e. the *Hpa*II-digested material showed amplification of the 45S promoter, similarly to the non-digested DNA.

Discussion

Epigenetic regulation of gene expression appears to be an attractive mechanism for the long-term memory of

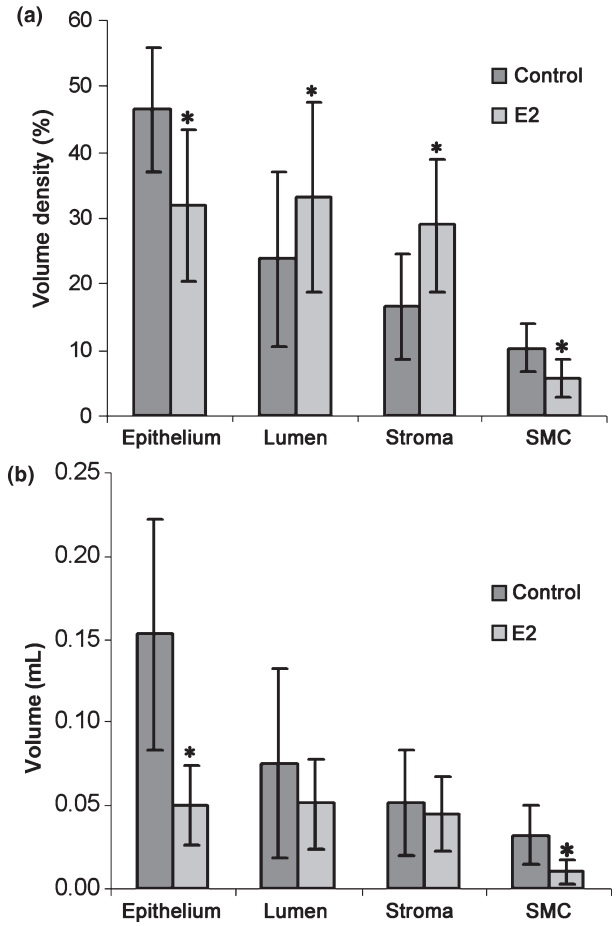

Figure 5 Stereological analyses of the rat ventral prostate in the control and oestrogenized rats. (a) Volume density measurement (%) and (b) volume (mL) of the epithelium, lumen, non-muscular stroma, and smooth muscle cells. The reduced volume density of the epithelium and smooth muscle cells is counterbalanced by increases in the organ fraction occupied by the lumen and stroma. The asterisks indicate statistically significant differences after two sample *t*-tests, at $p < 0.05$ (Control, $n = 3$; E2, $n = 5$).

developmental exposure to oestrogen and environmental oestrogen-related compounds (Prins, 2008; Tang *et al.*, 2008) and this was also shown to be true for the oestrogenized prostate (Ho *et al.*, 2006). We thought that it would be interesting to investigate what molecular mechanism would be responsible for regulating the functional state of the gland in oestrogenized animals.

To approach this question, we checked whether the mTOR pathway, which connects the energy status of the cell and its metabolic rate with the levels of macromolecular biosynthesis, was affected. Under normal metabolic conditions, mTOR phosphorylates and inhibits 4E-BP and allows protein synthesis to take place. We found reduced mTOR activity and hypo-phosphorylated 4E-BP, a condition associated with inhibition of protein synthesis (Pause *et al.*, 1994; Wullschleger *et al.*, 2006). Among other functions, mTOR also regulates ribosome biogenesis by sequestering TIF1A in the cytoplasm and inhibiting the formation of the rDNA transcription initiation complex based on RNA polymerase I (Mayer *et al.*, 2004).

Histological and morphometric analyses of the ventral prostate revealed important changes in the epithelial cells, which appeared shorter and exhibited compromising of the typical polarity related to secretory activity, as well as smaller and more compact nuclei, with a less evident nucleolus, compared with the controls. Stereology showed that the epithelium occupied a smaller fraction of the prostate tissue and a reduced volume in the whole gland. The imbalance in epithelium-stroma proportion in the gland has been described before, and likely results from an enhanced stromal and a reduced epithelial proliferation in response to oestrogenization (Prins, 1992). Moreover, nuclear morphometry revealed a reduced nuclear perimeter, area and standard deviation, confirming that the VP epithelial-cell nuclei were smaller and had more uniform and compact chromatin in oestrogenized animals.

The nucleolus of the epithelial cells in the prostate of oestrogenized rats is disintegrated, as shown by the increased number of silver-reactive dots and by the lack of correlation (and negative slope of the regression curve) between the nuclear area fraction occupied by argyrophilic material and the number of nuclear dots. This demonstrates the dissociation of individual NORs with no association of nucleolar RNA and proteins, the physiological meaning of which is the reduced activity of the nucleolus and an eventual reduction in ribosome biogenesis. As mentioned before, the use of histological sections might be inappropriate for the morphometric analysis

Table 1 Morphometric parameters of the ventral prostate epithelial cells in control and oestrogenized rats (E2)

	Nuclear area*	Perimeter*	Integration of optical densities*	Forma factor	StdDev
Control	5096 ± 180	275.8 ± 5.3	645120 ± 31688	0.84 ± 0.016	23.6 ± 0.6
E2	4208 ± 119	258.6 ± 5.9	485262 ± 7	0.79 ± 0.018	14.5 ± 0.3
	$p = 0.036$	$p = 0.007$	$p < 0.001$	$p = 0.009$	$P < 0.001$

Values are given as the mean ± standard deviation.
*Values are given in arbitrary units.

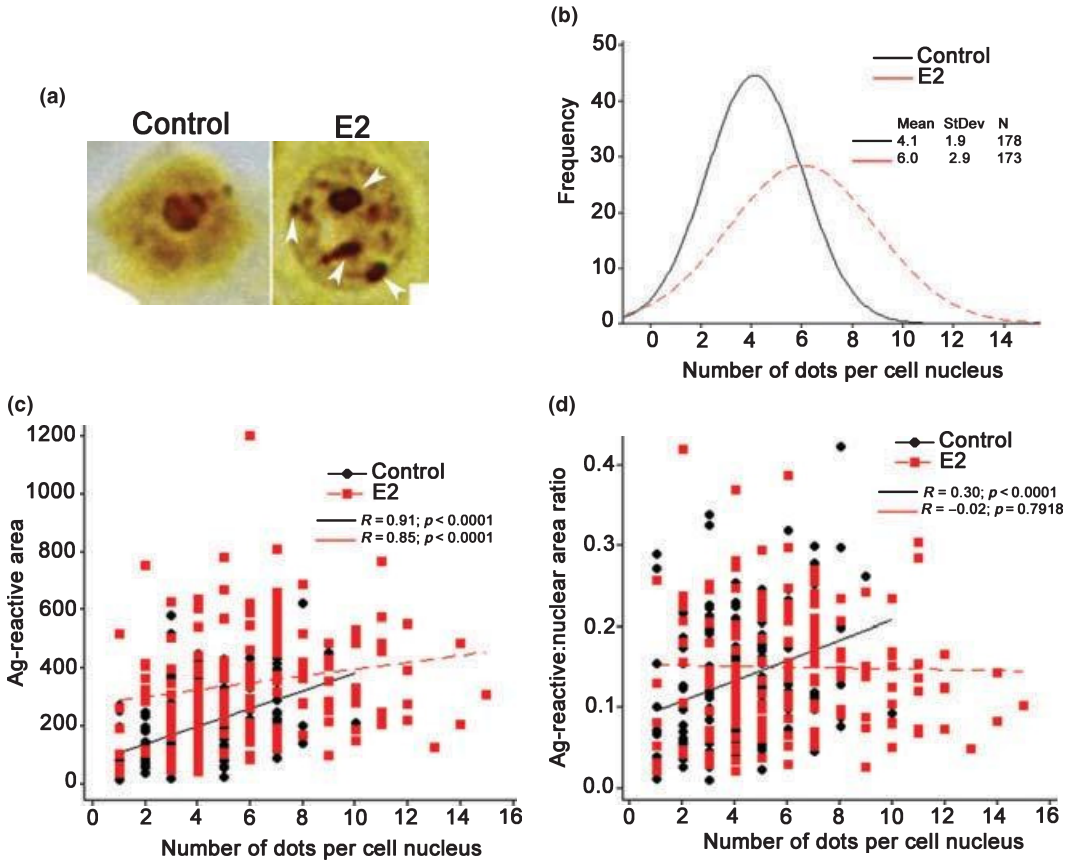

Figure 6 Morphometrics of the nucleolus in epithelial cells, and changes resulting from neonatal oestrogenization. (a) Representative images of the nucleolus in the epithelial cells of a control and oestrogenized animal after silver-impregnation, Bar = 10 μm. (b) Frequency distribution of the silver-reactive dots as identified by image analysis. (c) Scatter plot of the surface area of silver-reactive material with respect to the number of dots per individual nucleus in control (black circles) and oestrogenized animals (red squares). (d) Scatter plot of the nuclear area fraction occupied by the silver-reactive material with respect to the number of dots per individual cell nucleus. While a positive correlation was observed in the control (black circles), no increase in nuclear area fraction occupied by argyrophilic material was detected in oestrogenized animals (red squares).

of the nucleolus: it is less likely to affect the trends of the data obtained, as well as the maximum number of dots counted. This bias is also minimized by the use of correlative analyses between the measured parameters and other precautions (González-Pardo *et al.*, 1994). A further proof of the applicability of the present procedure is that the number of six NORs found in rat chromosomes 3, 11 and 12 (Kano *et al.*, 1976; Kano-Tanaka & Tanaka, 1982) is coincident with the mean number of dots found in oestrogenized rats. Similar nucleolar disruption was reported for DNMt1 deficient cells (Espada *et al.*, 2007). Furthermore, these aspects of the nucleolus are clearly correlated with the observed inactivation of the mTOR pathway.

The observed nuclear and nucleolar changes suggested a reduction in transcriptional activity. Consistently, we noticed that the amount of RNA extracted from the ventral prostate of oestrogenized rats was lower than that of the controls. To refine this observation, we applied a modified protocol for the simultaneous identification of DNA and RNA using acridine orange, and demonstrated that VP epithelial cells in the oestrogenized animals showed reduced RNA which appeared concentrated in the supranuclear region. Thus, nuclear compaction, nucleolar dismantling and RNA content were consistent with a generalized reduction in transcriptional activity.

In fact, we found that these parameters were associated with a greater increase of CpG methylation in the promoter region of the 45S rRNA precursor (estimated by the level of methylation of a single CCpGG site at position 142 bp of the transcription start), which is compatible with reduced transcriptional activity (Kunnath & Locker, 1982; Oakes *et al.*, 2003; McStay & Grummt, 2008; Murayama *et al.*, 2008).

It also seems relevant that transcription levels of rDNA are modulated by the energy status of the cell, by means of Sirt1 (Murayama *et al.*, 2008), resulting in altered

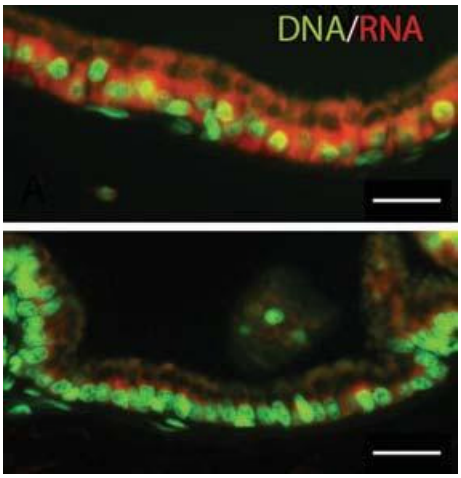

Figure 7 Simultaneous identification of DNA (green) and RNA (red) in the VP using a modified protocol for acridine orange-staining. There is a marked reduction in the amount of RNA produced by epithelial cells in oestrogenized animals, with a clear concentration in the supranuclear region. The absence of nuclear staining for RNA in the oestrogenized material can also be observed.

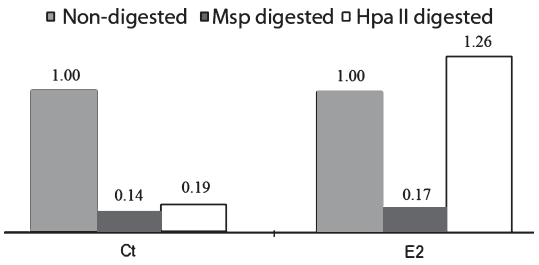

Figure 8 Genomic DNA extracted from isolated epithelial cells with or without digestion by Msp I (basal amplification) or Hpa II was amplified by qPCR, using a primer set for the β-actin gene as the internal control. The measurements demonstrated a residual 14–17% reaction after Msp I digestion (set as variations in the CCGG sequence among the hundreds of gene copies in the rat genome). Hpa II digestion revealed a minimum 5% methylation at the CCGG site in the promoter region of the 45S rDNA (calculated as the value obtained for Hpa II digested material minus that of the Msp digested material) and an almost complete resistance to Hpa II digestion in the same region after early postnatal oestrogenization (n = 3).

levels of rDNA methylation, although it remains to be determined whether they also result in nucleolar morphological and morphometric changes. Actually, Sirt1 is a histone deacetylase. Histone deacetylation is necessary for methylation, and both are related to transcription inactivation (Bernstein *et al.*, 2007). We note that these nuclear and nucleolar changes paralleled the decreased amount of phospho-mTOR and a corresponding reduction of phosphorylation of 4E-BP, which is thus free to inhibit protein synthesis initiation factor eIF4E (Pause *et al.*, 1994; Wullschleger *et al.*, 2006). Of note, both the PI3 kinase/Akt pathway and FRAP/mTOR kinase regulate 4E-BP1 activity.

It has been shown that the mechanism leading to the stable inhibition of gene transcription is based on epigenetic changes (Ho *et al.*, 2006). However, it is not known what sort of event redirects the epigenetic machinery/chromatin reorganization. It is possible that blocking one or many of the AR-regulated developmental signalling pathways responsible for prostate development (Pu *et al.*, 2007) might be involved. Moreover, it is intriguing that Sirt1 also modulates the action of insulin-like growth factor-1 (IGF-1), by blocking the production of IGF-binding protein-1, a pathway stimulated by oestrogens in stromal cells, at least in the mammary gland (Li *et al.*, 2007). It is tempting to speculate that a similar event might occur during early prostate development, and could represent a key element connecting oestrogen exposure with the modulation of mTOR, besides reinforcing the notion that oestrogen action takes place through oestrogen receptor α in the stroma, as demonstrated by Prins *et al.* (2001).

It remains to be investigated whether the observed structural and functional aspects of the ventral prostate have correlates immediately after E2 administration and later on before puberty, as it has been shown that the uterine changes in response to neonatal exposure to oestrogenic compounds are only established after exposure to pubertal hormones (Tang *et al.*, 2008).

In conclusion, E2 exposure in early postnatal life results in reducing gene expression, chromatin remodelling and compaction, reduced nucleolar activity and reduced ribosome synthesis, as well as blocked protein synthesis in prostate epithelial cells. It was clearly demonstrated by a general mechanism of gene repression, related to mTOR signalling and stabilized by epigenetic factors.

Acknowledgements

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4.3 MANUSCRITO III : *em elaboração*

"Neonatal exposure to high doses of 17–β–estradiol results in inhibition of heparanase–1 expression in the adult prostate"

Autores: Taize M. Augusto, Rafaela da Rosa Ribeiro and Hernandes F. Carvalho.

Department of Anatomy, Cell Biology, Physiology and Biophysics

(UNICAMP), Campinas, SP, Brazil.

Running Title: Heparanase–1 and neonatal estrogen

Correspondence: Hernandes F. Carvalho

Department of Anatomy, Cell Biology, Physiology and Biophysics – Institute of Biology

State University of Campinas (UNICAMP)

13083–863 Campinas, SP, Brazil

Tel. 55 19 3521–6118

Fax: 55 19 3521–6185

e–mail: hern@unicamp.br

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ABSTRACT

Heparanase–1 (HPSE–1) is an endoglycosidase that degrades heparan sulfate on the cell surface and extracellular matrix. The physiological functions of HPSE–1 include heparan sulfate turnover, embryo development, hair growth, and wound healing. It is implicated in a variety of pathologies, such as tumor growth, angiogenesis, metastasis and inflammation. HPSE–1 overexpression in a variety of malignant tumors suggests that it could be a target for anti–cancer therapy. HPSE–1 expression was transiently increased in the rat ventral prostate (VP) after castration. The promoter region of Hpse–1 gene have estrogen responsive elements (EREs), suggesting that the gene is regulated by estrogens. In this work, we have investigated the expression of HPSE–1 in the VP of 90–day–old rat after neonatal exposure to high dose estrogen. HPSE-1 was found by immunohistochemistry in the stroma but not in the epithelium of estrogenized animals. To determine whether inhibition of Hpse–1 expression in the epithelium was due to pre– or post–transcriptional regulation, epithelial cells were isolated by centrifugation in *Percoll* gradient and the presence of *Hpse*–1 mRNA was investigated by RT–PCR. The results showed that *Hpse*–1 mRNA was not detected in estrogenized animals. Considering that Hpse–1 transcription could be inhibited by DNA– methylation, we used the methylation sensitive restriction enzyme HpaII and RT–PCR to show that a single CCGG site at position +185 was more frequently methylated in the epithelium of estrogenized than in animals. Imunohistochemistry for 5–methyl cytidine revealed that the epithelial cell nuclei in estrogenized animals were heavily methylated. These results suggest that Hpse–1 expression was blocked in the epithelial cells of the VP by estrogen imprinting by a pre–transcriptional mechanism involving DNA methylation.

INTRODUCTION

It is long known that fetal exposure to maternal estrogen could cause epithelial squamous metaplasia of the human prostate and that it is reversed soon after birth as estrogen levels decrease (Driscoll & Taylor, 1980; Yonemura *et al.,* 1995). Low doses of estrogen during the gestation, in mice, increase the production of androgen receptor and the ventral prostate tissue in adult life (Nonneman *et al.,* 1992). In contrast, exposure to a high dose of estrogen compromises prostate growth, epithelial differentiation, secretory functions, and increases the incidence of prostatic intraepithelial neoplasia (PIN) and prostatitis (Prins *et al.,* 1992; Prins *et al*., 2001). This effect promoted by estrogen administration in the perinatal period was designated estrogen imprinting (Huang *et al.,* 2004).

The prostate gland is susceptible to estrogen imprinting, showing a reduced expression of the androgen receptor and inability to respond to androgen stimulus (Putz *et al*., 2001). Studies have demonstrated that estrogen exposure affects prostate metabolism, supporting the idea that the estrogenized prostates maintain a very low transcriptional and translational actitivities by affecting major signaling pathways and promoting nuclear compactation and nucleolar inactivation (Augusto *et al.,* 2010).

Heparanase–1 (HPSE–1) is a β–D–glucuronidase that cleaves heparan sulfate (HS) in specific sites. Its activity was associated with metastasis, neovascularization and inflammatory processes, given the fact that HS was an important component of basal membrane (Bernfield *et al.,* 1991; Iozzo & San Antonio, 2001; Kjellen & Lindahl, 1991; Murdoch *et al.,* 1992). HPSE–1 is highly expressed by tumor cells, and this overexpression favors the invasive phenotype (Ilan *et al.,* 2006). However, little is known about heparanase contribution to normal cells and tissue physiology.

Recently, studies have demonstrated that the expression of HPSE–1 was transiently increased after castration, showing a transition from epithelial to stromal cells and playing a key role in a second wave of epithelial cell death after castration (Augusto *et al.,* 2008; Augusto *et al.,* submitted).

Additional regulatory mechanisms can operate locally or systemically in the Hpse–1 transcription regulation. One example identified by Elkin *et al.* (2003) was the systemic regulator 17–β–estradiol. They identified responsive elements to estrogen in the Hpse–1 promoter region and demonstrated its functionality through assays with the luciferase reporter gene driven by Hpse–1 promoter.

In this work, we investigated how HPSE–1 was affected by high dose of estrogen exposure in neonatal life. The results presented here demonstrated that Hpse–1 expression was inhibited in the rat VP epithelial cells in adult life subjected to estrogen imprinting by a pre–transcriptional mechanism involving DNA methylation.

MATERIAL AND METHODS

ANIMALS

Male Wistar rats received 120 μg of 17 β–estradiol (E_2) in 25 μL corn oil (corresponding to 15 mg/kg body weight; mean animal body weight 8 g) at postnatal days 1, 3 and 5 (day of birth corresponding to day 0), to achieve most of the effects compromising ventral prostate growth, as described by Putz *et al.* (2001). Control animals received only the vehicle. These animals were kept under normal light conditions and received filtered tap water and Purina rodent chow ad libitum until postnatal day–90, when they were killed by anaesthetic overdose of xylazin and ketamin. The animal–handling and experimental protocols were approved by the University's Committee for Ethics in Animal Experimentation (Protocol no. 1271–1). Animals were weighed and their ventral prostate dissected out, weighed and either fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and used for paraffin or dissected for epithelial cell isolation (see below).

IMMUNOHISTOCHEMISTRY FOR HPSE–1

Paraformaldehyde–fixed paraffin–embedded tissues were cut into 5–μm sections, which were mounted on silane–treated slides, dewaxed in xylene, and rehydrated. Briefly, the sections were heated in a microwave oven in 100 mM citrate buffer pH 6, and then digested with 0.1 % pepsin in 0.01 N HCl for 20 min at room temperature. The sections were blocked with 3 % H_2O_2 for 10 min, followed by incubation with 1 % ABC kit serum (NCL–ABCu, Novocastra, Newcastle Upon Tyne, England) for 1 h and incubated overnight with the antibody against HPA–1 (C–20, Santa Cruz Biotechnology) (diluted 1:100). The tissue–bound

primary antibody was detected with the ABC kit (NCL–ABCu, Novocastra). The sections were counterstained with Harris hematoxylin.

IMMUNOHISTOCHEMISTRY FOR 5–METHYL CYTIDINE

Tissue sections obtained as above were washed once with PBS with 0.1 % Tween 20 (PBS–T) for 5 minutes and the sections were blocked with 3 % H_2O_2 for 15 min, followed by digestion with 0.2 % trypsin at 37°C for 10 min and treated with 2,5 N of HCl at 37°C for 15 minutes. The sections were washed three times in PBS–T. The blocking was done using 1 % of ABC kit serum in PBS (NCL–ABCu, Novocastra, Newcastle Upon Tyne, England) for 1 h and incubation overnight using the same blocking solution and the antibody against 5–Methyl cytidine (33D3) (Santa Cruz Biotechnology) (diluted 1:100). Negative controls were performed by omitting the primary antibody step. The tissue–bound primary antibody was detected with the ABC kit (NCL–ABCu, Novocastra). The sections were counterstained with methyl green. The nuclear staining was analysed by densitometry using the Imaje J software, version 1.33u (National Institute of Health, USA). The nuclear area was determined as the number of total pixels. The area covered by the immunohistochemical reaction product was determined after defining a threshold of 191. Results are shown as the mean area covered by reaction product and as nuclear area fraction (%) covered by the reaction product. For statistical analysis was used the T–test at $p < 0.05$.

PERCOLL ISOLATION OF VENTRAL PROSTATE EPITHELIAL CELLS

Epithelial cells from the ventral prostate were isolated using Percoll (Sigma Chemical Co., Saint Louis, MO, USA) gradient centrifugation as described by Liu at al. (1997). The

resulting preparations contained more than 95% of epithelial cells as determined by subsequent culturing and imunnohistochemistry for pan–cytokeratin (data not shown). Isolated cells from three animals per group were processed for DNA and RNA extraction.

GENOMIC DNA EXTRACTION

Genomic DNA was extracted from ventral prostate epithelial cells stored at −70 C and isolated by TNES method. Briefly, tissue samples were immersed in TNES buffer solution (Tris 250 mM pH 7.5, NaCl 2 M, EDTA 100 mM, SDS 2.5 %). The solution was then supplemented with proteinase K (10 mg/mL) and the samples were incubated for 5 h at 55 C. NaCl 5 M was added for protein precipitation. DNA was precipitated with isopropyl alcohol, washed with ethanol 70 %, resuspended in TE (Tris–HCl 10 mM, EDTA 1 mM, pH 8) and stored at −20 C.

PCR AMPLIFICATION OF HPSE–1 MRNA

Total RNA was extracted from isolated ventral prostate epithelial cells using Illustra RNAspin Mini kit (GE Healthcare, Germany) according to manufacturer's instructions. RNA purity was analyzed by the ratio 260/280 higher than 1.8 and by electrophoresis on 1.2% denaturing agarose gel to analyze the bands related to rRNA (28S and 18S). The RNA concentration was quantified by spectrophotometry in an Ultraspec 2100 pro apparatus (Amersham Biosciences). Total RNA (1 μg) was reverse–transcribed using SuperScript III First–Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's instructions. cDNA was quantified by spectrophotometry as described above. PCR amplification of cDNA was carried using the set of primers: (Hpse–1) Foward 5′–TAG TGC TGG CTC CCT TC–3′;

(Hpse–1) Reverse, 5′–AAC CTC TCT TCC CCA AA–3’, (β–actin) Forward: 5′–CTG GCC TCA CTG TCC ACC TT–3′; (β–actin) Reverse, 5′–AGT ACG ATG AGT CCG GCC C–3′.

For PCR reaction the kit Illustra PureTaq Ready–to–go PCR beads was used (GE Healthcare, Germany) with final reaction volume of 25 μL and 200 ng of cDNA.The following PCR thermal cycling conditions were used for the amplification of Hpse–1 and β–actin mRNA: a initial step of 94°C for 5 minutes and 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final cycle at 72°C for 7 min. The same conditions were used for β–actin, except that the annealing temperature used was changed to 62°C

Amplicons were electrophoresed on 2%, and the samples were pre–colored using 1 μL of GelRed 2x (Biotium, Inc., Hayward, CA). Gel images were captured and analyzed with a Kodak EDAS 290 Camera and Kodak Digital Science 1D 3.6 software (Eastman Kodak Company, Rochester, N.Y., USA).

PCR AMPLIFICATION OF GENOMIC DNA

Synthetic oligonucleotide primers (Fig 1) for genomic Hpse–1 were designed using Gene Runner 3.05 program and confirmed by BLAST search, as follow: Foward, 5′– TAA GGT ACG TGG ATA TTC AAA G –3′; Reverse, 5′– AAT GAA ATG TTC CCG GAT AC – 3′. The primer sets used for β–actin were the same used above. The following PCR thermal cycling conditions were used for the amplification of genomic Hpse–1: a initial step at 94°C of 15 min and 40 cycles at 94°C for 1 min, 55°C for 40 s, and 72°C for 2 min, and a final cycle at 72°C for 7 min. The reaction was composed of 100 ng of gDNA and Illustra PureTaq Ready–to–go PCR beads (GE Healthcare) in a final volume of 25 μL, according to manufacturer’s instructions. Amplicons were electrophoresed on 1% agarose gel, and the

samples were pre–colored using 1 µL of GelRed 2x (Biotium, Inc., Hayward, CA). Gel images were captured and analyzed with a Kodak EDAS 290 Camera and Kodak Digital Science 1D 3.6 software (Eastman Kodak Company, Rochester, N.Y., USA).

ANALYSIS OF THE METHYLATION STATUS OF A SINGLE SITE IN THE HPSE–1 GENE

To estimate the level of the genomic Hpse–1 methylation in epithelial cells, we applied a protocol based on the existence of a specific CCGG site at position +185 (according to the scheme described below). Restriction enzymes MSP–I and HPA–II (Fermentas Life Science, Burlington, Ontario, CA) recognize and digest de CCGG sequence, but HPA–II is only active if the site is non–methylated (Singer *et al.*, 1979). The set of primers used for Hpse–1 promoter region have inside the reverse primer the sequence CCGG. In summary the reaction was composed by 200 ng of genomic DNA and 0.2 µL of enzyme and was performed for 7 minutes followed by a PCR reaction for Hpse–1 gene.

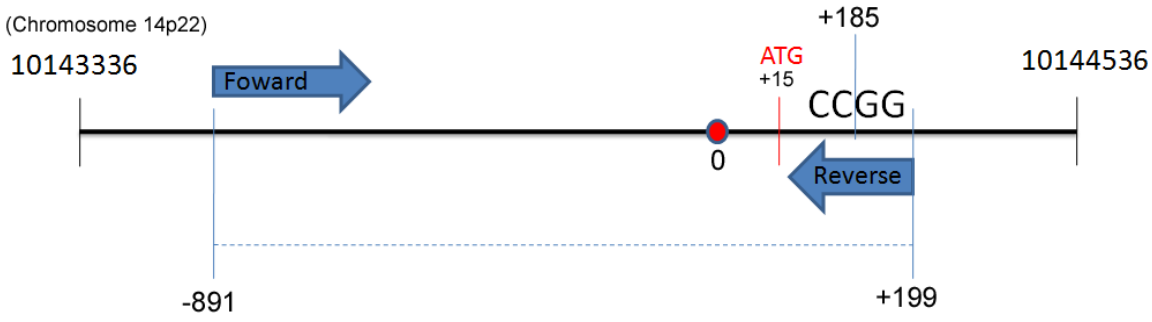

FIGURE 1 – *Scheme of a segment of the Hpse–1 gene from Rattus norvegiccus, showing the transcription initiation site (0), the position of the primers employed (-891, +199), and the position of the CCGG site studied (+185), based on chromosome 14p22 at access number (NC_005113.2, by NCBI database).*

RESULTS

ESTROGEN *IMPRINTING* COMPROMISED THE EXPRESSION OF HPSE–1 BY THE PROSTATIC EPITHELIAL CELLS

HPSE–1 was localized by immunohistochemistry in the epithelial cells of control animals (Fig. 2A, B). This reaction was lost in estrogenized animals (Fig. 2C, D). Some stromal cells for HPSE–1 also showed immune reactivity for HPSE–1 in estrogenized animals (Fig. 2C, D). In the control group, the HPSE–1 expression was concentrated in the region supranuclear assumed as occupied by Golgi apparatus.

Further analysis by RT–PCR demonstrated that no Hpse–1 mRNA was detected in the epithelial cells isolated from the VP of estrogenized animals (Fig. 3).

Considering that the results demonstrated the absence of Hpse–1 mRNA and protein which suggested a pre–transcriptional block, we decided to investigate whether DNA methylation could be involved. Investigations of the genomic Hpse–1 were done. A 1,09kb fragment from Hpse–1 gene (Fig 1), which includes a CCGG restriction site (exon 1) for cleavage of methylated specific enzymes MspI (Fig 4 B) and HpaII (Fig 4 C) were analyzed. Figure 4 demonstrated that the genomic DNA of estrogenized epithelial cells were methylated in the CCGG site at the position + 185 (Fig 1), showing that the exposure to high doses of estrogen in neonatal life regulate the epigenetic status of the genome, as demonstrated by the amplification of genomic Hpse–1 of the estrogenized epithelial cells after digestion with HpaII (Fig 4 C) (a specific enzyme that only degrades non–methylated CCGG sites).

In addition to observing that the Hpse-1 gene was methylated, since we have shown before that the 45S rDNA was heavily methylated in the epithelial cells of estrogenized animals (Augusto *et al.,* 2010) we then decided to investigate whether there was a global

increase in DNA methylation in imprinted epithelial cells. Immunolocalization for 5–methyl cytidine (Fig 5 A, B) showed that the nuclei of estrogenized animals (Fig 5B) were more intensily stained than in those of control animals (Fig 5A). Quantitative analysis demonstrated that the latter also showed larger area covered by the immunohistochemistry reaction product and that it covered a greater fraction of the nuclear area (Fig 5C).

DISCUSSION

Heparanase is an endoglucuronidase that degrades HS chains, a major glycosaminoglycan of cell surface, basement membranes and ECM. Its activity was associated with cell–cell and cell–ECM interactions and was first described in tumor progression. It is well known that tumor prostate cells and also normal rat prostate cells express heparanase–1 (Kosir *et. al,* 1997; Augusto *et al.,* 2008).

Recent studies showed that heparanase–1 expression was transiently increased after castration and that it contributed to a second peak of epithelial cell death in the prostate (Augusto *et al.,* 2008; Augusto *et al.,* submitted). In the present study, we found that, in contrast to the control, heparanase–1 mRNA and its protein were absent in the prostate epithelial cells of animals treated in the neonatal period with a high dose of estrogen. These results have suggested that the expression of heparanase–1 was blocked by a transcriptional mechanism.

Considering that the Hpse–1 gene might be regulated by DNA methylation in a series of circunstances and is particularly demethylated in tumors (Ogishima *et al.,* 2007; Shteper *et al.,* 2003) and that a series of genes were found to be differentially demethylated in response to estrogen imprinting (Prins *et al.,* 2008), we became interested to investigate

whether a variation in the DNA methylation was responsible for the transcriptional block in estrogen imprinted animals.

To approach the methylation status of the Hpse–1 gene, we designed an experiment that use methylation restricted enzymes, MspI and HpaII, which cleaves CCGG sites. MspI is capable to cleave CCGG site with or without methylation, while HpaII can only cleave non–methylated sites. Our experiment was based in the existence of a unique site at position +185. This strategy demonstrated that this unique site was more frequently methylated in the epithelial cells from the VP of estrogen imprinted animals than the controls. We have reported before that the 45S rDNA promoter was more frequently methylated in the imprinted animals and this was associated with nuclear compaction and nucleolar dismantling, in association with reduced translational activity (Augusto *et al.,* 2010).

Since DNA methylation is an important regulator of gene transcription and hypermethylation typically occurs at CpG islands mainly in the promoter region and is associated with gene inactivation, we decided to investigate whether a global hypermethylation was taking place in the epithelial cell nuclei of estrogenized animals. Results of the immunolocalization for 5–methyl cytidine revealed that estrogenized animals showed more intense staining than the control animals; with typically larger patches of chromatin and that the reaction product occupied a larger fraction of the nuclear area.

The present results demonstrated that Hpse–1 expression is inhibited in the epithelial cells of the VP in response to estrogen imprinting and that this is correlated with DNA methylation both locally at the Hpse–1 gene and globally in the DNA. This is

consistent with previous results from our laboratory which showed a more compact cell nucleus and increased methylation of the 45S rDNA (Augusto *et al.*, 2010). This is also consistent with regulatory mechanisms of Hpse–1 gene expression (Ilan *et al.,* 2006). It is also known that heparanase–1 promoter region has estrogen responsive elements site (EREs) (Elkin *et al.,* 2003). The present findings demonstrated that estrogen could also interfere indirectly with the expression of Hpse–1, regulating this gene by promoting epigenetic modifications. However, in contrast to the mammary gland, in which estrogen demonstrated a positive effect on Hpse–1 gene (Elkin *et al.,* 2003), the exposure to high estrogen concentration in the neonatal life results in repressive regulation of Hpse–1 gene in the rat VP.

It is possible that additional factors result in this overall inhibition of gene transcription, which involves global hypermethylation and chromatin compaction. One interesting point is that the estrogenized prostate results in early neoplasic growth as compared to the controls (Prins *et al*. 2007), while cancer development and progression have been associated with global hypomethylation (Foley *et al*., 2009). Conciliating these two pieces of evidence will be challenging.

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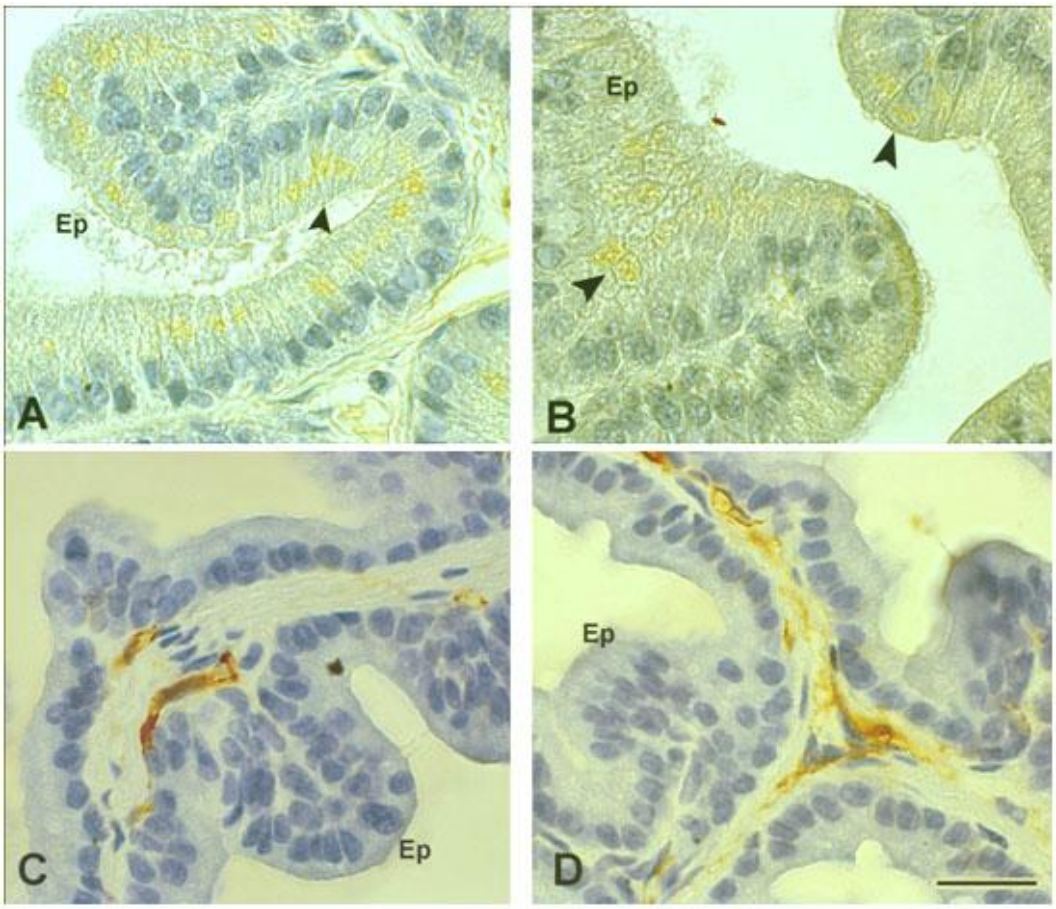
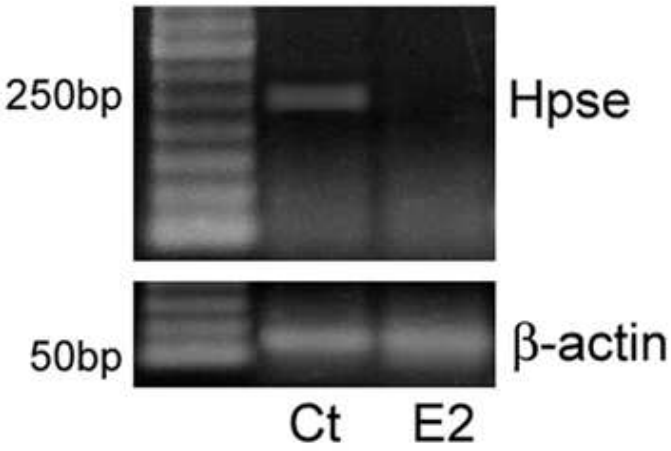
FIGURE 2



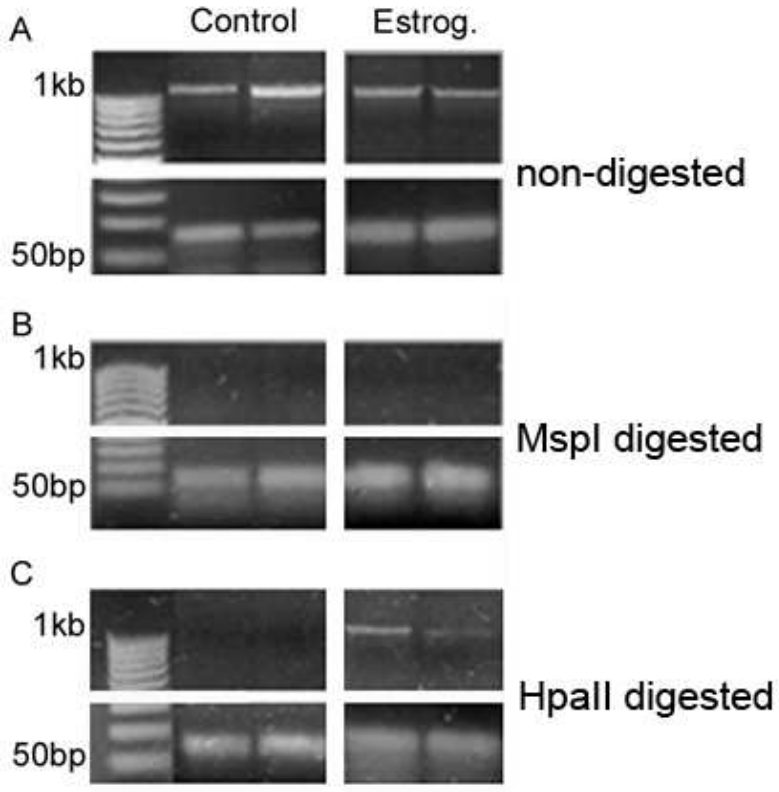
Immunohistochemistry for HPSE–1 in the ventral prostate of control (A, B) and estrogenized animals (C, D). The arrowheads indicate the reactivity for HPSE–1in the supranuclear region and in control animals, while no labeling were observed for the epithelial cells of estrogenized animals. Bar= 20μm.

FIGURE 3



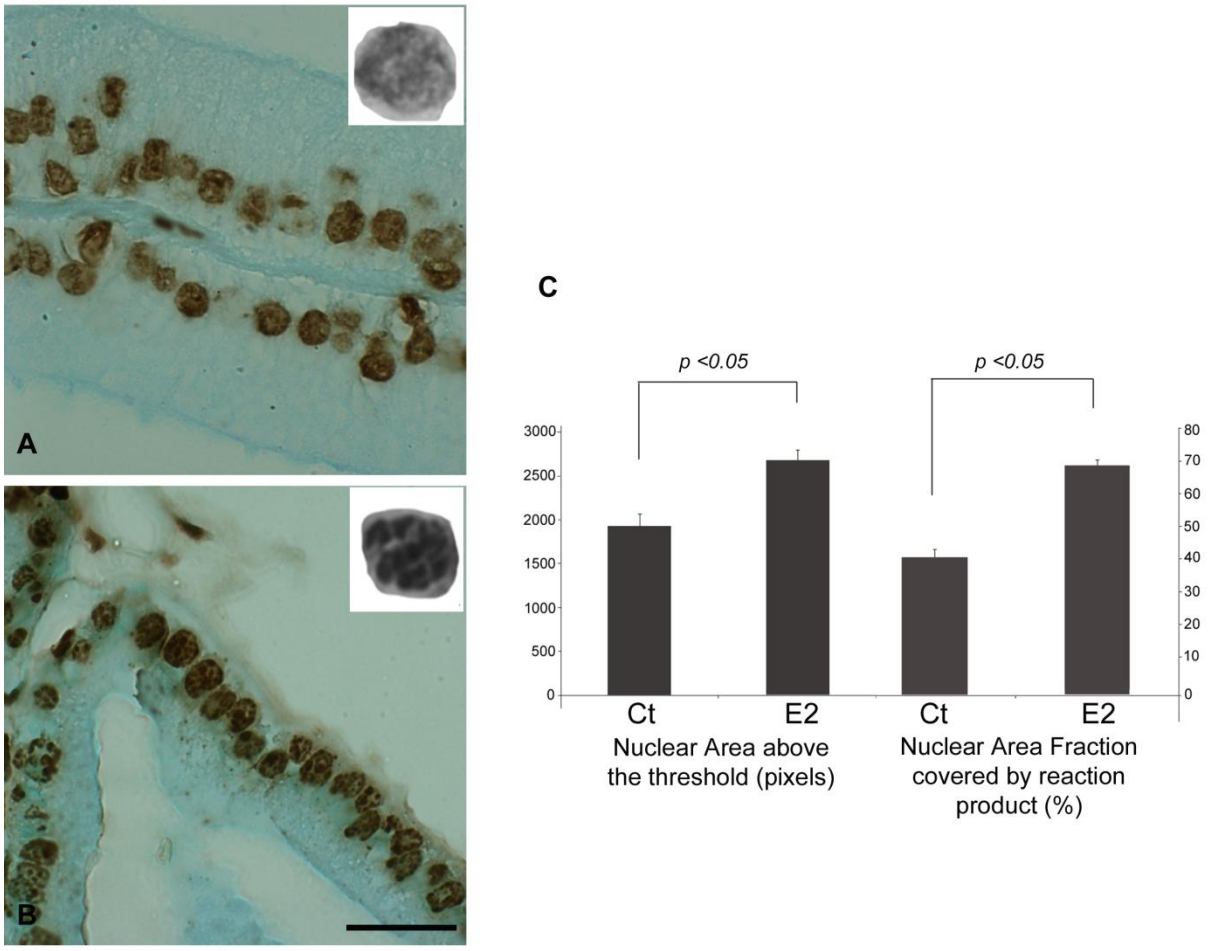
RT–PCR for Hpse–1 and β–actin in epithelia cells from control (Ct) and estrogenized animals (E2). RT–PCR reaction for Hpse–1 revealed a ~250 bp band in control epithelial cells and its absence in estrogenized animals. β–actin mRNA was used to demonstrate the cDNA integrity.

FIGURE 4



Restriction enzyme methylation analysis. (A) PCR reaction without digestion resulted in amplicons for both control and estrogenized epithelial cells. (B) MspI digestion. MspI cleaves CCGG sites irrespective of the methylation status. (C) HpaII digestion. HpaII identifies and cleaves non–methylated sites of CCGG. One could observe that the promoter region of estrogenized epithelial cells were resistant to HpaII, suggesting that it is more frequently methylated in estrogenized than the control cells.

FIGURE 5



5–Methyl cytidine reaction for control (A) and estrogenized (B) ventral prostates. Compared with the nuclei of the epithelial cells from control animals (A), the nuclei of estrogenized animals (B) were more intensily labeled, indicating a global hypermethylation status. (C) Analysis of the nuclear area (in pixels) above the threshold based in a threshold and the fraction (in percentage) of the labeling area per nuclear area. In detail (A–B), one could note the nuclear area measured for densitometry analysis. Bar A,B = 20μm.

5 CONCLUSÕES E DISCUSSÃO

A glândula prostática é altamente dependente de hormônios esteróides, majoritariamente de andrógenos. O estrógeno também desempenha papel fundamental, modulando a regulação androgênica. É notável como o desbalanço nos níveis de esteróides durante o início da vida pós–natal pode afetar o desenvolvimento, crescimento e afecções da próstata (Dryscoll & Taylor, 1980; Zondek & Zondek, 1980).

Em trabalho anterior (Augusto *et al*., 2008) foi observado que a castração promove a regressão da próstata ventral de ratos e que esta regressão está associada à diminuição no conteúdo de heparan sulfato e a aumento transitório da atividade enzimática da HPSE–1. Este achado levou à caracterização do papel da HPSE–1 na remodelação da próstata ventral de ratos que se dá a partir da castração, buscando paralelos com outras enzimas de remodelação da matriz extracelular, incluindo as relacionadas à degradação da membrana basal, e tendo como principal papel a adequação do estroma ao epitélio em regressão e a solubilização de fatores seqüestrados pelos componentes da MEC.

Bruni–Cardoso *et al.,* (2010) demonstraram sucessivas ondas de morte celular por apoptose na próstata ventral de ratos após a castração, com um segundo pico proeminente aos 11 dias de castração, tendo sido demonstrada uma íntima relação com a expressão das MMPs –2, –7 e –9 e a indução da apoptose neste período, uma vez que o tratamento com inibidores não específicos de MMPs (doxiciclina e hidrocortisona), assim como um inibidor que abrange inúmeras MMPs, o GM6001, diminui a freqüência de células epiteliais apoptóticas neste segundo pico. Estes resultados demonstram que a morte das

células epiteliais após a castração envolve uma complexa série de interações hormonais e parácrinas, resultantes da remodelação da matriz extracelular.

Neste trabalho, pudemos demonstrar que a HPSE–1 também desempenha papel relevante na segunda onda de morte das células epiteliais da próstata em regressão, com pico aos 11 dias. Assim como as MMP–2, –7 e –9, a HPSE–1 também tem sua expressão aumentada 24 horas antes do segundo pico apoptótico da próstata em regressão. Ensaios farmacológicos de inibição da HPSE–1 pela heparina isoladamente ou em combinação com o GM6001 diminuíram a taxa de morte de células epiteliais prostáticas, colocando a atividade da HPSE–1 dentre os fatores que contribuem para que ocorra a morte de células epiteliais por anoikis e uma conseqüente remodelação tecidual prostática pós–castração.

Além da testostona, o estrógeno também está presente na próstata atuando em sua fisiologia e manutenção.

Embora o *imprinting* estrogênico seja conhecido já há algum tempo, a caracterização dos seus efeitos sobre a próstata tem recebido relativamente pouca atenção. Nossa abordagem deste tema levou à demonstração de que o *imprinting* estrogênico leva à redução da ativação de mTOR e a hipo–fosforilação da 4E–BP, fatores associados com redução e/ou inibição da síntese protéica. Isto se dá em função da atividade de mTOR de conectar o nível energético celular, com a atividade metabólica, através da regulação de eventos como transcrição, síntese protéica e autofagia. mTOR também regula a biogênese ribossomal por inibir o complexo inicial de transcrição do DNA ribossomal (Mayer *et al.,* 2004).

Esta abordagem também permitiu demonstrar que este estado metabólico reduzido associa–se à diminuição da expressão gênica global, na maior compactação da cromatina, reduzida atividade nucleolar, e inibição da síntese protéica nas células epiteliais.

Embora o papel exato do estrógeno durante o desenvolvimento prostático ainda não seja conhecido, sabemos que a exposição a altas doses durante o desenvolvimento pode ocasionar doenças prostáticas observadas comumente na população masculina com o envelhecimento (Rajfer *et al*., 1978; Santti *et al*., 1994).

Com a idéia de que a alta exposição ao estrógeno no período neonatal ocasionaria uma diminuição global da transcrição gênica, foi investigado a expressão da HPSE–1, cuja função é a degradação de HS através da qual participa da remodelação tecidual, também importante por sua super–expressão em câncer. Estudos demonstraram que o promotor do gene da HPSE–1 possui elementos responsivos ao estrógeno (EREs), sugerindo que o gene é regulado pelo estrógeno (Elkin *et al.,* 2003), o que leva a crer que sua expressão pode estar alterada sob efeito do *imprinting* estrogênico.

Nossos resultados demonstraram que a exposição à alta concentração de estrógeno no período perinatal regula negativamente a expressão da HPSE–1 na idade adulta, ao contrário do que acontece em casos relatados como no endométrio humano (onde o estrógeno circulante na idade adulta regula positivamente a expressão deste gene) (Xu *et al*., 2007) ou em câncer de mama (Cohen *et al.,* 2007).

O *imprinting* estrogênico resultante reflete no bloqueio da expressão da HPSE–1, o que foi confirmado tanto em nível de proteína como no de RNA mensageiro, particularmente nas células epiteliais. Estes resultados sugeriram um bloqueio em nível transcricional. Dada a existência de informações de que o gene Hpse–1 pode ser regulado

por metilação do DNA, investigamos se a inibição observada poderia ser decorrente da hipermetilação. Os dados obtidos para um sítio CCGG específico, localizado na posição +181 com respeito ao início de transcrição demonstram haver maior freqüência de metilação nos animais estrogenizados do que nos animais controle. Esta observação é pertinente e coincide com a proposição de um mecanismo geral de inibição da transcrição nas células epiteliais prostáticas de animais estrogenizados, proposto anteriormente (Augusto *et al.,* 2010). Esta sugestão foi particularmente reforçada pela ocorrência de uma hipermetilação global do DNA nos núcleos das células epiteliais, conforme demonstrado pela imunohistoquímica para 5–metil citidina.

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Comissão de Ética na Experimentação Animal
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C E R T I F I C A D O

Certificamos que o Protocolo nº **1271-1**, sobre **"Papel da heparanase sobre a regressão prostática pós-castração: efeito da inibição por RNA de interferência e do imprint estrogênico"**, sob a responsabilidade de Prof. Dr. Hernandes Faustino de Carvalho / Taize Machado Augusto, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 28 de maio de 2007.

C E R T I F I C A T E

We certify that the protocol nº **1271-1**, entitled **"Role of heparanase in the prostatic remodeling after castration: interfering RNA and estrogen imprinting inhibition effects"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on May 28, 2007.

Campinas, 28 de maio de 2007.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEEA/IB – Unicamp
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

Telefone: (19) 3521-6359
Telefax: (19) 3521-6356
E-mail: comisib@unicamp.br
http://www.ib.unicamp.br/institucional/ceea/index.htm