UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

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"REGRESSÃO PROSTÁTICA PÓS-CASTRAÇÃO: CARACTERIZAÇÃO DAS ALTERAÇÕES CAUSADAS PELA PRIVAÇÃO ANDROGÊNICA E ALTA DOSE DE 17β-ESTRADIOL"

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Orientador: Prof. Dr. Hernandes Faustino de Carvalho

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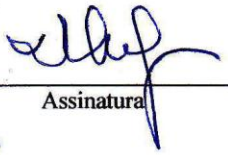

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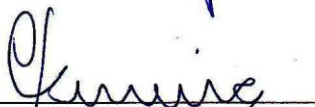

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Adormeci e sonhei que a vida era alegria;

despertei e vi que a vida era serviço;

servi e vi que o serviço era alegria.

(Rabindranath Tagore)

RESUMO

O desenvolvimento, a fisiologia e o câncer de próstata dependem de balanços entre os níveis de andrógenos e estrógenos, que agem via receptor de andrógenos (AR) e receptores de estrógeno (ERα e ERβ), respectivamente. Sabe-se que a cinética de morte das células epiteliais da próstata ventral de ratos (PV) após castração (grupo Cas), administração de alta dose de 17β-estradiol (grupo E2) e combinação de ambos (grupo Cas+E2) é diferenciada, com um nítido efeito aditivo nesta última situação (Garcia-Florez et al, 2005). Neste trabalho, procuramos investigar elementos comuns e exclusivos a estas diferentes condições hormonais, empregando análises morfológicas, estudo de componentes da via AKT/PTEN, e análise da expressão diferencial de genes (utilizando microarranjos de DNA) combinada com identificação de fatores de transcrição (FT) a ela relacionados. O peso relativo da PV foi significativamente diminuído nos grupos Cas e Cas+E2. A castração promove um padrão de descamação das células epiteliais que deve contribuir para redução do número de células epiteliais. No grupo E2, foi marcante a presença agregados protéicos citoplasmáticos e proliferação das células epiteliais com freqüente estratificação do epitélio. Outro aspecto importante foi o descolamento das células musculares lisas do epitélio quando este apresentava dobras epiteliais. No grupo Cas+E2, foram observados os diferentes aspectos observados em cada grupo individual. A análise bioquímica mostrou redução significativa na fosforilação de 4EBP2 nos grupos E2 e Cas+E2 (tendência similar foi observada no grupo Cas). Há uma grande quantidade de genes que são diferencialmente expressos em relação ao controle e que são comuns aos diferentes tratamentos. Surpreendentemente, não houve redução da expressão de probasina no grupo E2. A análise de ontologias e de termos enriquecidos mostrou a ausência de termos enriquecidos no conjunto de genes exclusivos do grupo Cas+E2, o que se concilia bem com a idéia de que os eventos observados neste grupo são aqueles observados nos dois grupos individuais. Em conclusão, a busca por sítios de ligação de FT na região promotora dos genes mais regulados em cada grupo e a identificação de FT nas rede de dos termos enriquecidos indicou os FT Evi-1, NF-Y, HNF-4, Elk-1, GATA-2, c-Rel, v-Myb e NFκB como candidatos a atuarem na regulação dos eventos associados à regressão prostática.

ABSTRACT

Prostate development, physiology and cancer depend on a fine balance between the levels of androgens and estrogens acting via the androgen receptor (AR) and the estrogen receptors (ERα e ERβ), respectively. It is known that the kinetics of apoptosis are different in the rat ventral prostate (VP) of castrated rats (Cas group) and in rats subjected to 17β-estradiol high dose (group E2) or their combination (group Cas+E2), with an evident additive effect in the latter situation (Garcia-Florez et al, 2005). In this work, we investigated elements in common and exclusive to each of these hormonal conditions, employing morphological analysis, components of the AKT/PTEN pathway and analyses of differential gene expression using DNA microarrays combined with a search for transcription factors (TF). The VP weight was significantly reduced in the Cas and Cas+E2 groups. In the E2 group, the remarkable effects were the presence of protein aggregates in the cytoplasm, and cell proliferation and layering of the epithelium. Another important aspect was the detachment of the smooth muscle cells from the epithelium when it showed infolds. In the Cas+E2 group, the different aspects observed in the individual groups were observed, except by less frequent epithelial layering. The biochemical analysis showed a significant reduction in 4EBP phosphorylation in the groups E2 and Cas+E2 (similar tendency was observed in the Cas group). There is a large number of differentially expressed genes as compared to the controls and shared by the different treatments, Surprisingly, there was no reduction in the expression of the probasin gene in the E2 group. The recovered gene onthologies and enrichment terms revealed the absence of enrichment terms among the genes exclusive to the Cas+E2 group, what conciliates with the Idea that the observed changes in this group are identical or at least very similar to those occurring in the individual treatments. In conclusion, the search for TF binding sites in the promoter region of the regulated genes and the identification of TF in the regulatory pathways obtained for the enrichement terms indicated the TF Evi-1, NF-Y, HNF-4, Elk-1, GATA-2, c-Rel, v-Myb and NFκB as candidates to regulate the events associated with prostate regression.

LISTA DE ABREVIATURAS

4EBP: do inglês, *Eukaryotic translation initiation factor 4E-1A-binding protein*

4EBP2: do inglês, *Eukaryotic translation initiation factor 4E-binding protein 2*

AKT: do inglês, *serine/threonine protein kinase Akt*

AR: receptor de andrógeno (sigla do inglês, androgen receptor)

ARE: elemento responsivo a androgénos (do inglês, androgen responsive element)

BCL2: do inglês, *B-cell CLL/lymphoma 2*

CAB: bloqueio combinado dos andrógenos (do inglês, combined androgen blockade)

Cas: grupo castrado por 72 horas

Cas: grupos de animais castrados

Cas+E2: grupo de animais castrados e que receberam alta dose de estrógeno por 72 horas

CD44: gliproteína de superfície cellular CD44

Ck: citoqueratina

CML: célula muscular lisa

DHT: dihidrotestosterona (5α-dihidrotestosterona ou 5α-androstan-17β-ol-3-one)

E2: grupo de animais tratados com alta dose de estrógeno por 72 horas

ERα: receptor de estrógeno alfa (do inglês, estrogen receptor alpha)

ERβ: receptor de estrógeno beta (do inglês, estrogen receptor beta)

FAS-L: do inglês, *Fas ligand (TNF superfamily, member 6)*

FLIP: do inglês, *flagellar biosynthesis protein*

HSP: do inglês, *heat shock protein*

MMP: metaloproteinase da matriz extracelular (do inglês, matrix metalloproteinase)

mTOR: do inglês, *mammalian target of rapamycin (serine/threonine kinase)*

NIP: neoplasia intraepitelial (sigla do inglês)

NR: nuclear receptor

p-4EBP2: do inglês, *phosphorylated eukaryotic translation initiation factor 4E-binding protein 2*

p-AKT: do inglês, *phosphorylated serine/threonine protein kinase Akt*

PD: próstata dorsolateral

PIN: neoplasia intra-epitelial (do inglês, prostate intraepithelial neoplasia)

PL: próstata lateral

PPARγ: do inglês, *peroxisome proliferator-activated receptor alpha*

p-PTEN: do inglês, *phosphorylated phosphatase and tensin homolog*

PSA: antígeno prostático específico (do inglês, prostate specific antigen)

PTEN: do inglês, *Phosphatase and tensin homolog*

PV: próstata ventral

SQM: metaplasia escamosa (do inglês, squamous metaplasia)

SUG: seio urogenital

T: testosterona

Vv%: densidade de volume

ÍNDICE

INTRODUÇÃO

A Próstata

A próstata é um órgão exclusivo do aparelho reprodutor de mamíferos. Ela é uma glândula exócrina que contribui com uma fração importante do líquido seminal incorporando íons citrato e zinco (que ajudam a neutralizar o pH ácido da vagina), frutose, lipídios estruturais, várias enzimas proteolíticas e substâncias anti-inflamatórias e imunossupressoras capacitando assim, a sobrevivência dos espermatozoides (Price, 1963).

Em ratos adultos a próstata é constituída por três diferentes lobos designados de lateral (PL), ventral (PV) e dorsal (PD) conforme a sua posição em relação à uretra (Figura 1), ao contrário da da próstata humana que não é lobulada. A próstata ventral possui o estrutura túbulo alveolar e é constituído por dois lóbulos que se encontram na ligação com a uretra. Cada lobo é formado por uma quantidade de estruturas epiteliais revestidas por uma única camada de células epiteliais (Jesik et al, 1982).

Vários componentes glandulares e não glandulares compõe a próstata (McNeal et al, 1988). O componente glandular é envolvido por um estroma muscular contrátil não glandular.

O epitélio prostático é formado por vários tipos celulares, diferenciados entre si por marcadores específicos. As células epiteliais luminares expressam citoqueratina (Ck) 8 e 18, junto com o antígeno prostático específico (PSA) (em humanos) e o receptor de andrógeno (AR). As células luminais basais, menos frequentes, possuem como marcadores as Cks 5 e 14, CD44 Bcl-2 e p63 (Schurr et al 1996, Wang et al, 2001).

Outro tipo celular presente no epitélio prostático são as células neuroendócrinas, as quais estão dispersas entre as camadas basal e luminal (Noordzij et al, 1995). Estas são caracterizadas pela independência androgênica (Abrahamsson, 1996) e são consideradas células totalmente diferenciadas sem capacidade proliferativa. A função exata destas células não está totalmente estabelecida, mas acredita-se que elas atuam no crescimento e diferenciação da glândula prostática, sendo também implicadas na origem de neoplasias (Sciarra et al, 2003).

Entre as células epiteliais e o estroma encontra-se a membrana basal. Esta estrutura é extremamente importante no controle das atividades celulares e, principalmente, na manutenção da fisiologia das células epiteliais (Hayward et al, 1998). Sabe-se que após a privação de andrógenos, e antecedendo os eventos apoptóticos, ocorre a perda de adesão da célula à

membrana basal, e que estas se tornam extremamente pregueadas e laminadas contendo moléculas intactas de laminina, mesmo 21 dias após a castração (Carvalho e Line, 1996).

As celular musculares lisas (CML) ocupam cerca de 5% do volume total da glândula e cerca de 14% do estroma na próstata ventral de ratos (Antonioli et al,2004). As CML têm ação preponderante nos mecanismos de estimulação parácrina, especialmente sobre o epitélio (Farnsworth, 1999), e provavelmente também sobre as demais células estromais.

A matriz extracelular do estroma prostático, além da membrana basal, é formada também por fibras de colágeno tipo I e tipo III, as quais sofrem um extenso rearranjo na próstata em regressão. Este rearranjo é caracterizado por um aspecto pregueado das fibras ao redor dos ductos e está intimamente associado às funções assumidas pelas células musculares lisas após a castração (Vilamaior et al, 2000)

Microfibrilas de colágeno tipo VI e fibras do sistema elástico também são encontradas e apresentam modificações durante a involução prostática. Estes componentes parecem estar envolvidos no controle de alguns aspectos do comportamento celular e na manutenção da integridade estrutural do órgão (Carvalho e Line, 1996; Carvalho et al,1997)

Na próstata ventral de ratos existe uma distinção morfológica dos ductos que estão mais na porção proximal (próximo à uretra), dos intermediários (que se encontram entre a região proximal e a distal) e dos ductos distais (mais distantes da uretra). Na região distal, é encontrada uma camada simples de células epiteliais colunares altas. Na região intermediária, as células epiteliais também são colunares altas, apresentando características de células secretoras, mas sem atividade proliferativa. Nesta região, a camada de células musculares lisas é fina e contínua. Já na região proximal, as células epiteliais são cúbicas e baixas, sendo freqüente a presença de células apoptóticas e as células musculares lisas formam uma camada espessa (Hayashi et al, 1991).

A formação dos ductos e a estruturação do epitélio e do estroma sofrem a ação principalmente do andrógeno, que é o principal hormônio esteroide responsável pelo controle do crescimento, proliferação, diferenciação e função da próstata (Leav et al, 2001; Cunha et al, 2002; Omoto et al, 2005).

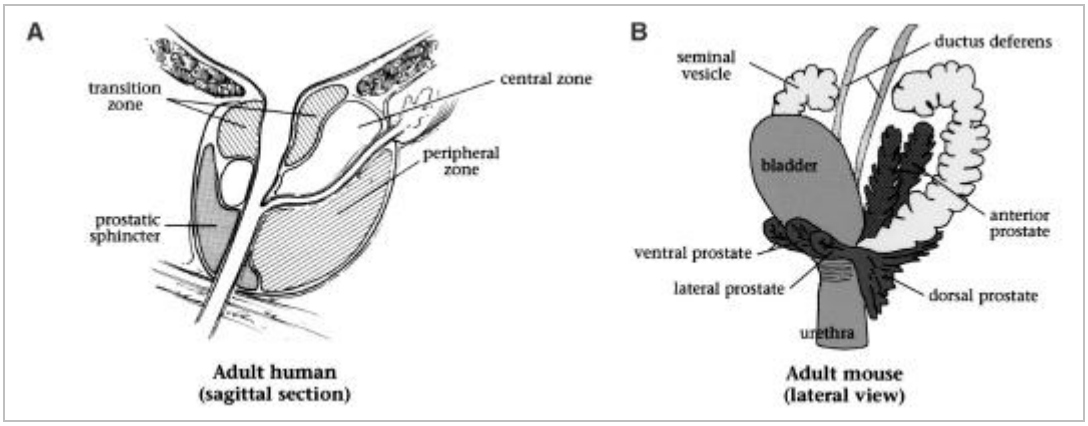

Figura 1: Esquema mostrando a localização e a anatomia da próstata humana (A) (McNeal, 1969) e da próstata de camundongo, com a caracteristica divisão em lóbulos (Cunha et al, 1987).

Desenvolvimento prostático

O desenvolvimento da próstata envolve os hormônios esteroides e intensa interação entre epitélio-estroma, a qual é mediada por fatores parácrinos. O primeiro tecido alvo de andrógenos é o mesênquima urogenital, o qual direciona o brotamento, ramificação e diferenciação epitelial. Por outro lado, o epitélio em desenvolvimento induz a diferenciação e o padrão morfológico do desenvolvimento das células musculares lisas. Portanto, é dito que essa interação entre epitélio e mesênquima/estroma é bidirecional (Cunha et al, 1987).

Seu desenvolvimento a partir do seio urogenital (SUG). Os primeiros sinais de formação da próstata são observados no 17º dia de desenvolvimento embrionário em camundongos, no 18º dia em ratos, e aproximadamente 10 semanas em humanos (Thomson, 2001), em resposta ao aparecimento de testosterona (T) produzida pelos testículos (Timms et al*,* 1994). O epitélio prostático é derivado da endoderme embrionária, ao contrário das estruturas Wolffianas tais como a vesícula seminal, ductos deferentes e epidídimos que são oriundos do mesoderma. As diferentes origens desses órgãos podem ter relevância no entendimento de mecanismos de crescimento diferentes entre próstata e órgãos derivados do ducto de Wollfian, embora todos mostrem crescimento dependente de andrógenos (Thomson, 2001). É importante ressaltar que, ao contrário da próstata, a vesícula seminal raramente desenvolve alterações proliferativas benignas ou malignas (Lee et al*,* 2007).

A ação androgênica pré-natal promove o brotamento prostático inicial (Timms et al, 1994). As subseqüentes morfogênese ductal, canalização e citodiferenciação epitelial também precisam de estimulação androgênica e estão associadas a um aumento perinatal transitório na

concentração de testosterona (Donjacour et al, 1988). Embora a testosterona (T) seja o principal andrógeno produzido pelo testículo, é a diidrotestosterona (DHT) que age na morfogênese prostática (Taplin et al,2001) através do receptor de andrógeno (AR).

A produção da T ocorre nos testículos (~95%) e na glândula adrenal (~5%) (Coffey, 1992). Além da DHT produzida pelos testículos, pode ocorrer a síntese local em diferentes tecidos, a partir da T, através da ação da enzima 5-α redutase (Andersson et al, 1991; Mahendroo et al, 2001). A DHT é um potente andrógeno, possuindo maior afinidade ao receptor de andrógeno (AR) do que a T propriamente dita (Aggarwal et al, 2010). O AR é o fator responsável pelos efeitos dos andrógenos na transcrição gênica. Nesse contexto, foi demonstrado que o uso de finasterida, um inibidor farmacológico da 5-α redutase, provoca a regressão da glândula prostática (Aggarwal et al, 2010).

Nosso laboratório demonstrou que a canalização do epitélio prostático em ratos depende da morte celular de células epiteliais em associação à polarização das células epiteliais luminais (Bruni-Cardoso e Carvalho, 2007) e que o crescimento epitelial é dependente da expressão localizada de MMP-2 e MMP-9 (Bruni-Cardoso et al, 2008).

Além do andrógeno, o desenvolvimento e o funcionamento prostático são modulados por hormônios somatotróficos (insulina, prolactina e hormônio do crescimento), ácido retinóico e estrógeno (Webber, 1981; Prins et al., 2001).

A interação entre os efeitos dos hormônios é também outro fator que deve ser ressaltado, alterando o comportamento das células prostáticas como, por exemplo, a interação da testosterona via AR com a via de sinalização da insulina causando a proliferação celular, no aumento e na diminuição do nível de cada hormônio resultando na instabilidade do equilíbrio celular (Vikran et al, 2011).

Ações dos andrógenos e o do estrógeno na próstata

O papel e o mecanismo de ação do andrógeno e de seu receptor tem sido estudado em uma variedade de órgãos alvo de andrógeno, incluindo a próstata. Além de ter um papel essencial no crescimento, desenvolvimento e proliferação das células prostáticas, também possui um papel importante no desenvolvimento e progressão do câncer de próstata.

O AR é um fator de transcrição nuclear e membro da superfamília de receptores de hormônios esteroides, a qual inclui receptores para estrógeno, progesterona, glicorticóide, minerolocorticóide, vitamina D, ácido retinóico e retinoide X (O'Malley et al, 1991).

Sua expressão acontece em duas diferentes isoformas: a predominante isoforma B (110 KDa) e a menos frequente, isoforma A (80 KDa). Ambas podem se ligar com T ou DHT (Gao et al, 1998; Wilson et al, 1995). Além disso, pode haver a ativação do AR através da sua fosforilação por proteínas quinases, sendo este um dos principais mecanismos de ativação ligante-independente por não necessitar da presença do hormônio (Lalevee, 2010).

Para que o processo de ativação ocorra, após a ligação do hormônio ao AR, este passa por uma mudança conformacional, dissocia-se de uma proteína HSP (*heat shock protein*) levando então, à dimerização do AR, sua fosforilação e translocação para o núcleo. Depois da ativação do AR através da ligação de T ou DHT, há a ligação desse receptor com co-reguladores, que cooperam entre si ativando ou inativando a transcrição de diversos genes, pela ligação aos elementos responsivos ao andrógeno (ARE) presentes na região promotora de genes que são regulados por andrógeno (Heinlein e Chang, 2004; Griekspoor et al, 2007; Koocheckpour, 2010).

Os receptores nucleares em geral fazem parte da superfamília de *Nuclear hormone receptor* (NR) os quais se caracterizam por funcionarem como fatores de transcrição que agem através de fatores corregulatórios não-ligantes ao DNA, incluindo co-ativadores e co-repressores, na região cis-regulatória do genes alvo (Wang et al, 2007). Mais de 300 proteínas tem sido identificadas como moduladoras da atividade do AR (Culig e Santer, 2011).

Com base na sua funções primárias melhor conhecidas, os co-ativadores e co-repressores podem ser agrupados em: (1) complexos de remodelação de cromatina; (2) modificadores das histonas; (3) componentes da via de ubiquitinação/proteosoma; (4) componentes da via de sumoilação; (5) proteínas envolvidas nos mecanismos de splicing e metabolismo de RNA; (6) proteínas envolvidas no reparo de DNA; (7) Charperonas e co-charperonas; (8) proteínas do citoesqueleto; (9) proteínas envolvidas na endocitose; (10) adaptadores, scaffolds, transdutores e integradores das vias de sinalização; (11) reguladores do cico celular; (12) reguladores de apoptose; (13) oncoproteínas virais dentre outras sem categorias bem definidas (Heemers e Tindall, 2007).

O papel do AR como um fator de transcrição vem sendo estudado para que assim possa ser compreendido o seu papel na regulação de genes importantes que são expressos no câncer de próstata (Tabela 1).

Tabela 1. Funções do receptor do AR estudadas pelas técnicas de CHIP-seq ou CHIP-on-CHIP métodos de estudo das modificações da cromatina e a identidade de genes-alvo (Adaptado de Itkone e Mills, 2011).

Modelo	Método	Conclusão	Referência
VCap,LNCaP e amostras de tecidos	ChIP-Seq para AR e H3K27me3 (marca para repressão transcricional)	ERG reprime a atividade do AR através de um mecanismo que envolve aumento de H3K27me3 dependente de EZH2 em loci dos genes específicos.	(Yu et al, 2010)
Linhagens celulares de LNCaP dependente ou independente de andrógeno	Arrays de expressão gênica com e sem AR knockdown e tratamento com hormônio CHIP/CHIP-on-chip para AR Phosphor-ser5 RNA POLIII na atividade dos promotores ativos FoxA1, GATA2, MED1 e Oct1 como fatores de transcrição colaboradores H3K4me1, H34me2 como marcodores de enhancer Transfecção de KDM1 para enhancer demetilado	O AR regula a expressão de genes do ciclo celular em câncer de próstata andrógeno-independente. UBEC2 é tomado como um exemplo e um mecanismo dependente do involvimento de FOXA1 como enhancer	(Wang et al, 2009)
LNCaP	ChIP-on-chip com arranjos de expressão de promotores	Identificação de elementos resposivos ao andrógeno (AREs) não-canônicos Coenriquecimento de fatores de transcrição ETS predizendo co-associações de ETS no promoter e validado por ETS-1	(Massie et al, 2007)
LNCaP	Tilling array para ChIP-on-chip nos cromossomos 21 e 22	Identificação de AREs não-canônico e de Oct-1, GATA2 e FoxA1 como fatores de transcrição colaboradores pioneiros (FoxA1)	(Wang et al, 2007)
HPr-1AR (derivado de células epiteliais de próstata humana imortalizada e expressando tipo selvagem de AR)	Arranjo de expressão para time course de tratamento com andrógeno e NimbleGen microarray de tiled-oligonucleotídeo costomizado (~104 kb regiões genômicas centradas no sítios de início da transrição dos 548 genes candidatos responsivo ao hormônios) por ChiP-on –chip de AR	Inferência do novo gene alvo de AR e discussão da necessidade clusters de AREs para predição de dependência de andrógeno e alguns casos associados com clusters genicos	(Bolton et al, 2007)

O AR é expresso na próstata de roedores adultos normais na totalidade dos núcleos das células epiteliais luminais e em parte das células epiteliais basais. As células esromais, especialmente as CML são também AR positivas, porém expressam menor quantidade de AR (Mirosevich et al, 1999). Ao longo dos ductos não existe uma diferença significativa da expressão do AR no sentido proximal-distal, o que sugere que as diferentes respostas a envolvem

hormônios esteróides são medidas indiretamente por fatores parácrinos e não pela expressão regional diferenciada do AR (Prins et al, 1992).

O andrógeno tem papel preponderante no câncer de próstata. Apesar de não ser um composto carcinogênico, a ação do andrógeno e seu estado funcional são importantes mediadores do desenvolvimento do câncer de próstata. Adicionalmente, homens castrados quando jovens não desenvolvem câncer de próstata (Miyamoto et al, 2004).

A privação androgênica promove a diminuição da proliferação de células epiteliais da próstata ventral de ratos. O bloqueio da produção do andrógeno por orquiectomia resulta em uma perda de 80% do total de células da próstata ventral 10 dias depois da cirurgia. Além disso, os níveis de testosterona caem para menos de 10% comparado ao controle depois de duas horas da cirurgia (Isaacs, 1984). Dessa maneira, a involução da próstata seguida à castração tem sido extensivamente utilizada como modelo para estudos da fisiologia da próstata.

Tal involução é compreendida em um complexo processo fisiológico caracterizado pela perda de peso prostático, apoptose das células epiteliais e uma inversão de uma estrutura predominantemente glandular para outra dominada pelo estroma. Esta involução envolve uma significativa remodelação tecidual, a qual inclui a remodelação do estroma e a eliminação das células apoptóticas (Kyprianou e Isaacs, 1988; Powell et al 1996; Carvalho e Line, 1996; Carvalho et al, 1997; Powell et al, 1999; Vilamaior et al, 2000; Augusto et al, 2008).

O ER, assim como o AR, pertence à família dos receptores nucleares e apresenta dois subtipos, ERα e ERβ, que podem ter papéis fisiológicos distintos (Altundag et al, 2004). Os dois receptores compartilham homologia entre si, mas são produtos de diferentes genes (Yang et al, 2003). Foi demonstrado que o silenciamento do ER através da metilação do seu promotor pode estar envolvido na hiperplasia prostática benigna e no desenvolvimento do câncer de próstata (Li et al, 2000).

Além do andrógeno, o estrógeno (17β-estradiol) exerce papeis sistêmico e local na próstata. Sua ação mais conhecida é como um anti-andrógeno por bloquear a produção hipotalâmica do hormônio liberador de gonadotrofina e assim inibir a produção de testosterona pelos testículos. Um efeito local desse hormônio é mediado pelos seus receptores na próstata, ERα e ERβ os quais possuem diferentes distribuições (Kuiper et al, 1997). No animal adulto o ERα é expresso no estroma e o ERβ expresso principalmente no epitélio (Weihua et al, 2002).

Além da produção natural do estrógeno, há também a possível conversão do andrógeno circulante em estrógeno através da enzima aromatase em vários tecidos periféricos tais como: células granulosas, células de Leydig e células de Sertoli, neurônios, pré-adipócitos, condrócitos, osteócitos, células musculares lisas vasculares (Simpson et al, 1999). Em camundongos *knockout* para a enzima aromatase, verificaram-se várias mudanças prejudiciais tanto em machos quanto em fêmeas, como acúmulo de gordura, hiperinsulinemia e resistência a insulina. Porém, somente em machos foi possível identificar esteatose hepática a qual é revertida quando os animais são tratados com estradiol (Czajka-Oraniec e Simpson, 2010).

Estudos mostraram que a dosagem de estrógeno à qual a próstata é exposta no período neonatal resulta em diferentes consequências. Uma breve exposição de roedores a estrógenos durante o desenvolvimento neonatal provoca um efeito irreversível e dose dependente na morfologia, organização celular e função prostática (Prins et al, 1992). Exposição a baixas doses de estrógeno durante a gestação em camundongos provoca aumento do peso da próstata no adulto, redução dos níveis de receptor de andrógeno e também um aumento do brotamento (Nonneman et al, 1992).

Já, se a dosagem do estrógeno for alta durante o período neonatal, ocorre um "*imprinting*" permanente que inclui reduzido crescimento prostático, defeitos na diferenciação epitelial, função secretora alterada e displasia associada à idade, semelhante à neoplasia intraepitelial (NIP) (Prins, 1992; Prins et al, 2001). Acredita-se que esse "*imprinting*" causado pela ação estrogênica na próstata seja devido a alterações nas concentrações de andrógeno via a inativação eixo hipotálamo-hipófise-gonodal ou a um efeito local na próstata (Huang et al, 2004).

O "*imprinting*" promove um mecanismo geral de inibição da transcrição, com alterações da organização da cromatina e do nucléolo, com baixa expressão dos rDNA por metilação da região promotora, além de um mecanismo global de inibição da síntese proteica baseada na inibição da via mTOR/4EBP (Augusto et al, 2010). Além disso, a expressão da Heparanase-1, uma proteína importante para processos de metástase, processos inflamatórios, é inibida nas células epiteliais da próstata ventral de ratos adultos que foram submetidos a alta dose de estrógeno quando recém-nascidos por um mecanismo pré-transcricional envolvendo a metilação do DNA (Augusto et al, 2011).

A interação de andrógenos e estrógenos é demonstrada pela frequência, co-localização dos ER e AR tanto nas células epiteliais como nas células estromais, sugerindo uma regulação fina entre concentração hormonais e expressão dos receptores (Garcia-Florez, 2005).

Em um trabalho realizado em nosso laboratório demonstrou-se que a cinética de morte celular apoptótica difere entre animais castrados, animais que receberam alta dose de 17β-estradiol e animais castrados e tratados com 17β-estradiol, sugerindo a existência de mecanismos diferentes de indução de apoptose nas células epiteliais da próstata ventral pela privação androgênica e pela alta dosagem de estrógenos (Garcia-Florez et al, 2005) (Figura 2).

Muito pouco se sabe das vias de sinalização relacionadas à morte das células epiteliais prostáticas em resposta à privação androgênica. Um trabalho específico demonstrou que a ação da MMP-7 levaria à liberação de FAS-L da matriz, o que levaria à ativação da via extrínseca de morte celular (Powel et al, 1999). Entretanto, não fomos capazes de detectar em ratos Wistar o aumento detectado na expressão de MMP-7 por aquele grupo (Bruni- Cardoso et at, 2010).

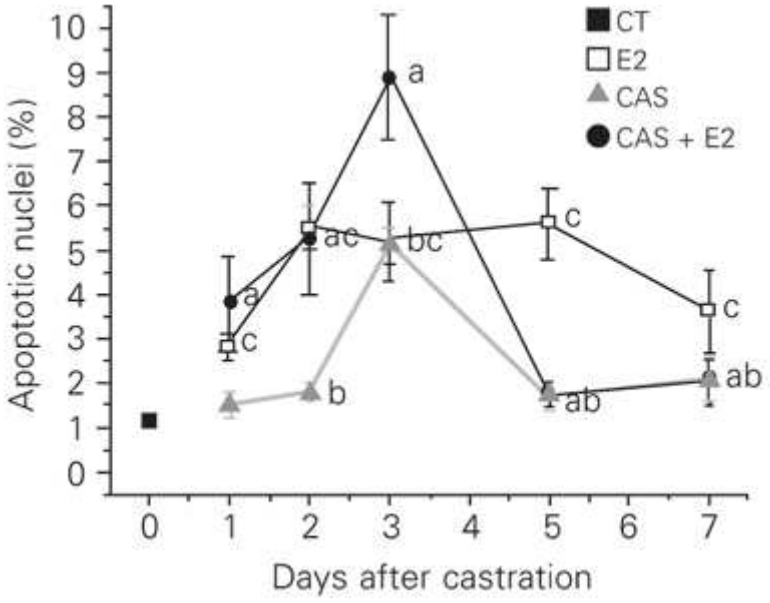

Figura 2. Cinética de apoptose de células epiteliais prostáticas em resposta à castração (Cas), à administração de 17β-estradiol (E2) e à combinação de ambos (Cas+E2) (Garcia-Florez et al, 2005).

Por outro lado, tem sido sugerido que as alterações vasculares resultantes da castração (aumento de permeabilidade vascular, restrição de suprimento sangüíneo e morte das células

endoteliais) levaria a uma restrição de nutrientes e hipóxia, que resultaria na morte das células epiteliais (Buttyan et al, 2000; Shabsigh et al, 2001). Adicionalmente, já foi demonstrado que a expressão do receptor de andrógeno não é elemento relevante na indução da morte das células epiteliais pela privação androgênica, o que levou os autores a sugerirem a existência de mecanismos parácrinos indutores da morte celular (Kurita et al, 2001). Embora tenha sido demonstrado que o inibidor de caspase-8 FLIP (proteína inibitória FLICE-*like*) seja regulado por andrógeno e possa, por esta razão, estar envolvido na morte das células epiteliais (Nastiuk e Krolewski, 2008), os processos envolvidos continuam desconhecidos.

O câncer de próstata

A motivação para a investigação da regulação do crescimento e fisiologia prostática dá-se pela existência de várias complicações patológicas que afetam essa glândula, sendo a próstata o sítio de vários tipos de inflamações e de alterações proliferativas benignas e malignas, que se apresentam principalmente com o envelhecimento (Pfau et al, 1980).

O carcinoma prostático é uma das mais importantes doenças malignas diagnosticadas nos homens, principalmente a partir dos 50 anos. 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. Isto se deve ao fato de que a maioria dos tumores de próstata origina-se das células epiteliais glandulares da região periférica da próstata, as quais são dependentes de andrógenos para sobreviver e proliferar (Cunha et al, 1987). Muitas abordagens têm sido usadas para o tratamento do câncer de próstata: castração cirúrgica a qual, apesar de ser barata pode causar uma série de efeitos colaterais ao paciente (Fossa et al, 1994; Clark et al, 2001); castração por medicamento com estrógeno semi-sintético (DES) (Huggins et al, 1941) fitoestrógenos (Castle et al, 2002), antagonista de LH-RH (Denis et al, 1998; Seidenfeld et al, 2000); bloqueio combinado dos andrógenos (CAB) a qual neutraliza o andrógeno oriundo do testículo e da glândula adrenal do rim (Miyamoto et al, 2004); monoterapia antiandrogênica com flutamida, nilutamida, ou bicalutamida, e outros (Verhelst et al, 1994) (Figura 3).

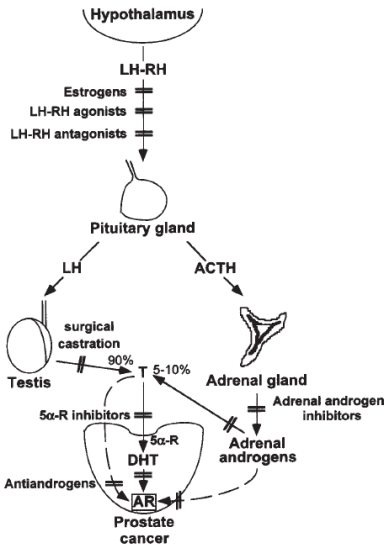

Figura 3. Estratégias para terapia hormonal. LH-RH liberação do hormônio lutenizante; ACTH-hormônio adrenocorticotrópico; T-testosterona; 5-αR- 5 alfa redutase; DHT-diidrotestosterona; AR-receptor de andrógeno (Miyamoto et al, 2004).

A instalação de um câncer independente de andrógeno também é conhecida sendo esta forma letal e com capacidade metastática (Feldman e Feldman, 2001). Esta forma de câncer parece estar associada com a modificação genética na qual algumas células são selecionadas dentre aquelas que conseguem continuar vivas sem o andrógeno. Alguns estudos apontam que uma forma de modificar tais células e torná-las selecionadas, através da terapia com anti-andrógeno flutamina, por exemplo, o qual pode selecionar mutantes para AR e acaba agindo como um agonista e não antagonista (Taplin et al, 1999). Além disso, outras diversas teorias apontam para um aumento de testosterona circulante para compensar o declínio geral de circulação do hormônio (Labrie et al, 1986).

Além do andrógeno o estrógeno também tem um papel na promoção de câncer de próstata. O estrógeno sozinho ou em sinergismo com o andrógeno, é indutor de um crescimento aberrante e transformações neoplásicas na próstata (Ho, 2003).

Sabe-se que a administração de estrógeno em doses farmacológicas induz o desenvolvimento de lesões com capacidade proliferativa chamada metaplasia escamosa tanto na próstata como em outros órgãos (Yonemura et al, 1995; Cunha et al, 2001; Risbridger et al, 2001). Essa alteração se caracteriza pela presença de um epitélio estratificado, presença de citoqueratina 10 e atividade proliferativa aumentada (Risbridger et al, 2001).

A mudança metaplásica inicia-se com proliferação das células epiteliais basais, as quais sequencialmente se diferenciam em células escamosas. Em ratos, a administração de estrógeno em conjunto com o andrógeno levou ao desenvolvimento de uma lesão pré-câncer similar àquela vista na neoplasia intra epitelial (PIN) observada em humanos e uma alta incidência de adenocarcinoma prostático (Wong et al, 1998; Tam et al, 2000).

Desta forma, o conhecimento das possíveis vias de indução de morte das células epiteliais prostáticas no contexto da presença ou ausência de estímulo androgênico, parece ser relevante.

JUSTIFICATIVA

Dada a relevância da privação androgênica na regulação da fisiologia da próstata e no tratamento do câncer prostático, parece-nos interessante compreender os aspectos teciduais, celulares e moleculares associados à regressão do órgão em resposta à orquiectomia, ao tratamento com alta dose de 17β-estradiol e à combinação dos dois, procurando identificar mecanismos que colaboram com a inativação do receptor de andrógeno.

OBJETIVOS

1. Caracterizar morfologicamente os efeitos da privação androgênica, alta dose de estrógeno e combinação de ambos os tratamentos na próstata ventral de ratos;
2. Identificar proteínas relacionadas à inibição dos mecanismos de sobrevivência, proliferação e morte celular na próstata ventral de ratos induzida por privação androgênica e pela administração de estrógeno e possíveis conexões com as vias gerais de regulação do metabolismo celular;
3. Identificar fatores de transcrição candidatos à contribuirem na regulação da expressão de genes envolvidos na resposta à privação androgênica e a alta dose de estrógeno.

Os resultados dessa tese serão apresentados na forma de dois capítulos escritos no formato de manuscritos a serem submetidos a publicação.

1. Capítulo 1: Androgen deprivation and 17β-estradiol high dose promote a series of morphological changes unique to each treatment and a common reduction in protein synthesis indicated by decreased 4EBP hypophosphorylation in the rat ventral prostate.

2. Capítulo 2: Identification of candidate transcription factors for regulating the rat ventral prostate gland response to androgen deprivation and high dose 17β-estradiol administration.

Androgen deprivation and 17β-estradiol high dose promote morphological changes unique to each treatment and reduced protein synthesis activity associated with 4EBP hypophosphorylation in the rat ventral prostate

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ABSTRACT

Androgens are key regulators of prostate physiology. Their effects are modulated by estrogen among other hormones. Estrogen acts systemically as a strong anti-androgen and locally in the prostate gland via estrogen receptors. This work was undertaken to characterize the morphological changes associated with androgen deprivation and 17β-estradiol. In this work we have examined the response of rat ventral prostate to castration (Cas group), 17β-estradiol high dose (E2 group) and their combined treatment (Cas+E2 group) by examining the histological changes and the involvement of the AKT/PTEN signaling pathway, given its role in cell survival/death. The novel finding in the Cas group was the presence of desquamation of groups of epithelial cells. In the E2 group, epithelial cell layering associated with the presence of apoptotic and mitotic cells in the epithelium, (related to squamous cell metaplasia), the formation of protein aggregates in the cytoplasm and detachment of the smooth muscle cells in areas of epithelial infolding. The associated treatment (Cas+E2 group) resulted in less frequent epithelial layering, but presented apoptotic and mitotic cells in the epithelial layer, epithelial cell desquamation and the formation of protein aggregates in the cytoplasm of the epithelial cells. Western blotting for different proteins in the AKT/PTEN pathway showed a significant reduction in the p-4EBP/4EBP ratio, indicating a marked reduction in protein synthesis. In conclusion, the effects of high dose 17β-estradiol in the prostate gland differs from those expected from its sole anti-androgen effect, and consist in a series of locally induced alterations, likely via ERα. The observed differences might well affect the relapse of androgen-independent prostate cancer in human patients subjected to either chemical or surgical castration, as they induce differential behavior of the epithelial cells, at least transiently.

INTRODUCTION

Androgens are required for prostate development, growth and physiology (Timms et al, 1994). The subsequent ductal morphogenesis, canalization, and epithelial differentiation also depend on androgen stimulation, resulting from a transient increase in testosterone levels in the perinatal period (Donjacour et al, 1988). In the normal adult rat prostate gland, androgen receptor (AR), the nuclear receptor that binds the androgens testosterone and dihydrotestosterone, is expressed in both epithelial and stromal cells (Mirosevich et al, 1999; Prins et al, 1992).

There are different studies pointing to the importance of AR in promoting prostate cancer by regulating prostate cell growth. Surgical or chemical castration is first line therapy to promote prostate tumor regression. In rats, the testosterone levels decrease to less than 10% of the control as quickly as two hours after castration. The resulting weight loss is associated with about 66% and 80% of the total ventral prostatic cells being lost at 7 and 10 days after surgery, respectively (Isaacs, 1984; Kyprianou and Isaacs, 1988). A classic peak of prostatic epithelial cell apoptosis occurs three days after castration (Garcia-Florez et al, 2005), which is much later than the drop in circulating and tissue concentrations of both testosterone and dihydrotestosterone (Kashiwagi et al, 2005). It is also known that events related to blood supply, blood vessel permeability and endothelial cell death takes place as earlier as 18-20 hours after castration (Franck-Lisbrant et al, 1998; Shabisgh et al, 1999). We have shown that a series of events take place in prostate gland at different time points under androgen-deprivation (Vilamaior et al, 2000; Felisbino et al, 2007; Peters et al, 2010), in particular a metalloproteinase-dependent second wave of epithelial apoptosis at day 11 (Bruni-Cardoso et al, 2010).

Besides androgens, estrogen exerts systemic and local effects on the prostate. Estrogen acts an anti-androgen effect by blocking the hypothalamic production of gonadotropin-releasing hormone and thereby inhibiting the production of testosterone by the testis. The local effects of estrogens are mediated by the estrogen receptors ER-α and ER-β, which have distinctive tissue distributions (Kuiper et al, 1997). In adult rats, ER-β predominates in the epithelium and ER-α is preferentially expressed in stromal cells, including the smooth muscle cells (Weihua et al, 2001). When neonatal rat ventral prostate was treated with testosterone plus estrogen in culture, where the testosterone levels are kept the same, the growth was retarded and the apoptosis incidence was significantly increased as compared with cultures treated with testosterone alone (Jarred et al, 2000; Taylor et al, 2006). Studies using ERα knockout (ERαKO) mouse prostates have

consistently shown that ERα is involved in the prostatic branching morphogenesis and is required for normal prostate development (Chen et al, 2009). It has also been shown that ERβ regulates prostate growth and hyperplasia by binding both 17β-estradiol or the testosterone metabolite 3betaAdiol (Weihua et al, 2001, 2002). As the levels of androgens decrease with age, it has been suggested that the varying androgen/estrogen ratios would orchestrate much of the aging related changes, including the incidence of prostate cancer, in particular due to the unbalance in the expression of genes related to oxidative stress (Pang et al, 2002).

Rats and mice exposure to estrogen hormone in the perinatal or neonatal period (estrogen imprint) leads to reduced responsiveness to androgens in adulthood and increased incidence of severe dysplasia at early age in the prostate gland (Prins et al, 1997; Prins et al, 2001; Augusto et al, 2010). In contrast, exposure of adult prostate to estrogens leads to squamous metaplasia (SQM) characterized by epithelial layering, cytokeratin 10 expression, and increased proliferation (Risbridger et al, 2001). The SQM seems to be associated with ERα, since the ERα knockout mouse (pes-ERαKO) is resistant to SQM transition in response to diethylstilbestrol an ER agonist (Chen et al, 2012).

Previously, we found different epithelial cell apoptosis kinetics in the rat ventral prostate by using different androgen/estrogen ratios (Garcia-Florez et al, 2005). It appeared important to us because either castration or estrogen might be used as surgical or chemical castration, respectively. Even though they result in prostate regression, there could exist characteristics individual to either treatment.

We have hypothesized that distinct morphological and/or biochemical changes would result from different levels of androgen and estrogens. To characterize these changes we have performed a high resolution light microscopy study and overviewed elements of AKT/PTEN signaling pathway, a central regulator of energy status, metabolism and cell proliferation/death decisions, in the rat ventral prostate in response to androgen deprivation by surgical castration (Cas group) (low androgen, low estrogen), high estrogen exposure (E2 group) (falling androgen level and high estrogen) and a combination of both (Cas+E2 group) (low androgen, high estrogen). The results reveal a series of novel findings on the morphological aspects associated with castration, as well as a general inhibition of protein synthesis, as indicated by 4EBP hypophosphorylation. In addition, they showed that the short term 17β-estradiol high dose promoted signs of squamous metaplasia, including epithelial cell proliferation, apoptosis and

layering, detachment of the smooth muscle cells from the epithelial structures, and cytoplasmic protein aggregate formation. We have also observed the persistence of cell proliferation in both latter groups.

MATERIAL AND METHODS

Animas and treatments

Twenty-four 21-day-old male Wistar rats were obtained from the Multidisciplinary Center for Biological Research (CEMIB), State University of Campinas. The animals were kept under normal light (12 hours cycle) conditions and received filtered tap water and Purina rodent chow *ad libitum*.

At the 90^{th} day after birth, the rats were divided into four groups (n=6) and assigned to different treatment groups. Animals in the first group were castrated (Cas group; low androgen, low estrogen) by orchiectomy via scrotal incision with ketamine (150 mg/Kg body weight) and xylazin (10 mg/kg body weight) anesthesia. Animals in the second group received one high dose 17β-estradiol (E_2 group; high estrogen) diluted in corn oil (25 mg/Kg body weight). The third group received a combination of orchiectomy and one high dose 17β-estradiol (Cas+E_2 group; low androgen, high estrogen). In the control group (Ct; high androgen, low estrogen) animals received only the vehicle (Garcia-Florez et al, 2005). Three days after the treatments, the rats were killed by anesthetic overdose, and the ventral prostates were dissected out, weighed and fixed for histology (n=3) or snap frozen in liquid N_2 before processed for the biochemical analysis (n=3). The relative weight was determined by the ratio between the gland weight and total body weight.

Fixation and histological processing

Ventral prostates were cut in small fragments. The fragments were immediately fixed in 2.5% glutaraldehyde plus 0.25% tannic acid in Millonig's buffer pH 7.2 for 24 hours, washed in distilled water, dehydrated and embedded in historesin. Two-micrometer thick sections were cut with glass knives and stained with hematoxylin and eosin. Analyses were performed using a Zeiss Axioskop and images were captured with an Zeiss Axiocam MRc CCD camera.

Stereology

Stereology was performed according to Huttunen et al (1981) and used before (Garcia-Florez et al, 2005; Antonioli et al, 2007). In brief, ten microscopic fields per animal (n=3 animals in each experimental group) were superimposed with a grid containing 65 lines and 130 points. The volume densities (Vv%) of epithelium, lumen, stroma and smooth muscle cells were calculated as the percentage of points hitting each tissue compartments and expressed as percentage of the total volume of the gland. The volume of these compartments was calculated by multiplying the corresponding Vv% for each compartment by the weight of the gland weighed before fixation and was expressed in microliters. Results were presented as the means ± standard deviations. One way ANOVA followed by the Tukey's multi-comparison test were performed to determine differences between the groups. Statistical significance was set at $p<0.05$.

Western blotting

Thirty milligrams of prostates fragments (n=3 for each experimental group) were incubated for 10 min in 270 μL of phosphatase blocking buffer (10 mM EDTA, 100 mM Trizma base, 10 mM sodium pyrofosfate, 100 mM sodium fluoride, sodium orthovanadate, 2 mM PMSF, 0.1 mg/mL aprotinin in water) before the addition of 30 μL Triton X-100 and further incubation for additional 10 min. Protein concentration in the extracts was determined by the Bradford's dye binding essay (Bio Rad, Hercules CA, USA) (Bradford, 1976) using a BSA standard concentration curve. Fifty μg protein were electrophoresed under reducing conditions in 10% (for AKT) or 12.5% (for the other proteins) acrylamide gel at 100V. The proteins were then transferred to nitrocellulose membranes (Amersham Bioscience) at the fixed 400 mA current. The immunoblotting steps included three washes with 0.1% Tween-20 in TBS (TBS-T) and protein block with 5% skim milk (PPARγ, p53, AKT, p-AKT) or 5% BSA (p21, p-4EBP2, p-histone H3, p-PTEN, PTEN) in TBS-T. The primary antibodies employed were rabbit polyclonal anti-PPARγ (cat. 516555; Calbiochem, Germany), rabbit polyclonal anti-phospho-histone H3 (cat. 9713), rabbit polyclonal anti-phospho-4EBP2 (cat. 2855); rabbit polyclonal anti-p21 (cat. 2947); rabbit polyclonal anti-PTEN (cat. 9559) and rabbit polyclonal anti-phospho-PTEN (cat. 9549) purchased from Cell Signaling (Beverly MA, USA), rabbit polyclonal anti-phospho-AKT (cat. sc7985-R), goat polyclonal anti-AKT (cat. sc1619), mouse monoclonal anti-p53 (cat. sc55747) and rabbit polyclonal anti-actin (cat. sc1616) purchased from Santa Cruz Biotechnology (Santa Cruz CA, USA), and rabbit polyclonal anti-4EBP2 (cat. G21234) purchased from

Invitrogen (Grand Island NY, USA). The antibodies were diluted 1:1000 in the corresponding block solution but with reduced (3%) concentration of the blocking agent, except for the anti-actin, which was diluted 1:2000 in TBS-T. The membranes were incubated with the primary antibodies overnight at 4ºC, washed three times in TBS-T and then incubated with the corresponding peroxidase-conjugated goat anti–rabbit IgG (cat. G21234; Invitrogen), rabbit anti-goat IgG (cat. A5420; Sigma-Aldrich, Carlsbad CA, USA) or goat anti-mouse IgG (cat. AP124P, Millipore, Billerica MA, USA) diluted 1:2000 in the same solution as the primary antibody for one hour at room temperature.

The peroxidase reaction was observed using in the Western Blotting Luminol Reagent (cat. SC2048, Santa Cruz Biotechnology) and the image was acquired with the G-Box using the GeneSnap software (Syngene, Frederick MD, USA). The intensity of the bands was determined by densitometry using the Image J software and the results were analyzed by ANOVA followed by the Tukey's *post hoc* multi-comparison test. $p< 0.05$ indicated significant differences between the means.

RESULTS

Weight variation

Castration (Cas group) promoted a significant reduction in the rat ventral prostate relative weight (calculated as the ratio between the prostate weight and the animal body weight) as compared to the control (0.057±0.02 *vs* 0.076±0.01, $p<0.05$). There was no weight variation in the relative weight of the ventral prostate from rats of the E2 group as compared to the control (0.072±0.02 *vs* 0.076±0.01). The combined treatment (Cas+E2 group) resulted in a significant reduction of the prostate relative weight as compared to controls (0.059±0.01 *vs* 0.076±0.01, $p<0.05$), that was very similar to that observed in the Cas group.

Histological aspects

The major aspects of the ventral prostate gland histology are shown in Figure 1A-D. The classical tubulo-acinar structure is observed (Fig. 1A). The proximal region depicts a short cylindrical cell layer, with a clear cytoplasm and large cell nucleus (Fig. 1B). The intermediate region presents a single layer of cylindrical secretory cells with supranuclear less colored region (Fig. 1C). The distal region is composed by taller cells with ellyptical cell nuclei (Fig 1D). The stroma is scarce, with smooth cells, fibroblasts and many individual cells including mast cells.

The effects of castration (Figs. 1E-H) are evident even under low magnification. The major event observed is the presence of desquamation of the epithelial cell lining (Fig. 1E). Apoptotic cells are observed in the proximal, intermediate and distal regions. The epithelial cells in the proximal region are shrunk and intensely stained (Fig. 1F). Detached groups of epithelial cells were frequent in the lumen. The intermediate regions show various changes in staining intensity and nuclear compaction in addition to the typical apoptotic cells (Fig. 1G). Aspects of apocrine secretion are observed (Fig. 2A). No changes were seen in the smooth muscle cells. Cells in the distal region appear shorter and less compact than in the control group (Figs. 1H and 2C). The desquamated cells showed various aspects of nuclear compaction and a clear halo around the cell nucleus (Fig. 2B). One as hitherto not described observation was the swelling of the apical part of the cytoplasm in the epithelial cells (Fig. 2D).

In the E2 treatment group (Figs. 1I-L), two major aspects were observed at low magnification. The first was the rupture of the association between the smooth muscle cell layer and the epithelium where the epithelium presented a number of infoldings (Fig. 1I). The second was the layering of the epithelial cells (Fig. 1K).

The proximal region presented cells very similar to the control group, in terms of size and staining, but apoptotic cells were clearly identified. Frequent images of macrophage-like cells phagocytosing apoptotic cells were found in the epithelial layer along the ductal system (Figs. 2E and G). Mitotic figures were readly seen (Fig. 1J and inset). The intermediate region presented a multilayered arrangement of short cells and many aspects of protein accumulation in the cytoplasm (Fig. 1K). Apoptotic and mitotic cells were found in the distal region, among short cylindrical cells (Fig. 1L). Protein aggregation was found in the cytoplasm of epithelial cells in the distal region (Fig. 2F). Cell desquamation was absent.

Most of the signs observed in the Cas and E2 groups were observed in the Cas+E2 group (Figs. 1M-P), including epithelial lining desquamation, epithelial infolds and detachment from the underlying smooth muscle cells. Cells in the proximal region were shorter, intensely stained and presented compact nuclei. Protein aggregates were easily seen (Figs. 1N, 2H and I). In the intermediate region, there were apoptotic cells and mitotic cells, but the signs of layering were missing (Fig. 1O). Apoptotic and mitotic cells were also found in the distal region (Fig. 1P) and macrophage-like cells appeared with large phagocytosis bodies (Figs. 2H and I). Protein aggregates were frequent in the cytoplasm of the epithelial cells (Figs. 2H and I). Figure 2J shows

a large area of epithelial cell desquamation, along with adjacent to well preserved epithelium. Epithelial layering associated with squamous metaplasia was much less evident than in the E2 group.

Stereology

The epithelium, lumen, stroma and smooth muscle cells occupied about 32%, 33%, 20% and 15% of the gland volume, respectively (Figs 3A-D). Three days of androgen deprivation did not change the Vv% of the four tissue compartments. In the E2 group, there was a decrease in the lumen and an increase in the Vv% of the stroma and smooth muscle cells, compared to the controls. Combined Cas+E2 group promoted a significant reduction in the Vv% of the epithelium as compared to the control.

The stereological determination of compartment volumes showed reductions in the volume of the epithelium (Fig. 3E) and the smooth muscle cells (Figs 3H). The luminal volume was reduced in the E2 group, but remained unchanged in the other groups as compared to the controls (Fig. 3F). After three days of androgen deprivation by castration (Cas group), there was a significant reduction in the volume of the stroma (Fig. 3G).

Metabolic markers and signaling

Western blotting was used to identify differences in the metabolic state of the gland as indicated by the activity of the AKT/PTEN pathway. Representative images of the Western blots for AKT, PTEN, 4EBP and their phosphorylated forms, PPARγ, p53, p21 and phospho-histone H3 are presented in Figure 4. Though some differences were observed among the experimental groups, the quantitative data obtained after normalization with total actin, which was used a loading control, showed no significant variation, except for phospho-4EBP/4EBP ratio in the Cas and Cas+E2 groups (Fig. 5). A reduction was also seen in the E2 group, which was though not significant.

DISCUSSION

The present investigation demonstrates that androgen deprivation and 17β-estradiol promote quite distinct effects on the rat ventral gland structure that contribute to the weight loss in addition to inducing apoptosis and promoting an overall inhibition of protein synthesis, which

were common to both treatments. It also shows that changes exclusive to each treatment were reproduced independently when both treatments were combined.

The prostate gland is a classical example of hormone-regulated organ in which androgen deprivation promotes epithelial cell death and regressive changes in the epithelium (Kerr and Searle, 1973). As the epithelium regresses, stromal cells and extracellular matrix are remodeled to adjust to the reduced organ size (Vilamaior et al., 2000; Antonioli et al, 2004; 2007).

Androgens exert both antagonist effect on apoptosis and agonist effect on cell proliferation (Isaacs, 1984) and androgen deprivation by either surgical or chemical castration is first line of treatment for prostate cancer. Estrogen has anti-androgen effects by inhibiting the hypothalamic release of gonadotropin-releasing hormones. Both surgical castration and 17β-estradiol administration have been substituted by second and third generation anti-androgen therapies, which alleviate most of the side effects of each one, such as feminization and cardiovascular problems but at increased treatment costs (Piper et al, 2002).

Even though epithelial apoptosis is a major event promoting prostate regression, other factors contribute to the gland regressive changes such as reduced blood flow and endothelial cell apoptosis (Franck-Lissbrant et al, 1998; Shabisgh et al, 1999), elimination of the secretion accumulated in the lumen, reduced cell proliferation and increased compaction of the stroma, including atrophy of the smooth muscle cells (Antonioli et al., 2005; 2007).

Since 17β-estradiol exhibit local effects in the gland, we became interested in determining whether it would show additional effects besides those promoted by acting as an anti-androgen. In a previous study (Garcia-Florez et al, 2005), we showed that 17β-estradiol high dose promoted apoptosis with a different kinetic as compared to androgen deprivation. As a matter of fact, epithelial cell death was induced even earlier than the resulting androgen depletion. Nonetheless, we have also shown that simultaneous castration and high dose 17β-estradiol showed apparent additive effects in terms of the number of apoptotic cells up to day three after the beginning of the treatment, which led us to suggest that they promoted cell death by distinct mechanisms. Here, we have decided to extend our understanding of the mechanisms leading to altered kinetics and addictive effects of both androgen deprivation and high dose estrogen, reproducing the same protocol as employed before and examining morphological characteristics of the gland at day three after the beginning of the treatment and by performing a biochemical analysis of the AKT/PTEN signaling pathway.

The morphological analysis revealed interesting findings. In the Cas group, the most prominent and novel finding was the desquamation of epithelial cells groups. We have observed this before, but it was usually attributed to low preservation during fixation. This possible artifact was discarded in the present study because we have associated careful handling, glutaraldeyde fixation and resin embedding to achieve maximum tissue preservation. In concert with this idea, the changes observed in the desquamed cells (picnotic nuclei and a clear cytoplasmic halo around them) could not result from inappropriate processing. Furthermore, epithelial cell desquamation was not observed in the experimental groups that did not involve androgen deprivation (control and E2 groups). We have recently shown the existence of a second peak of apoptosis taking place at day 11 after castration, which was distinct from the classical peak at day 3 by the dependence on the expression and activation of MMP-2, MMP-7 and MMP-9 (Bruni-Cardoso et al, 2010). It seems though that this desquamation is dependent on other proteolytic enzymes affecting cell adhesion. Cathepsin D is one such possible candidate, which were long ago described as being activated by castration (Wilson et al, 1991). We have also found that MMP-15 (membrane-type MMP-2) expression is up-regulated in response to the experimental treatments employed in this present work (see accompanying manuscript) and might well be involved in this process. Also, the observation of apocrine secretion (which seems to result from an accelerated rate of secretion and apical cytoplasmic swelling) contributes to the novelty of the present results.

The changes observed in the E2 group were also novel and not observed as a result of the anti-androgen effect of estrogens. The major findings were epithelial layering, mitotic cells in the epithelial layer, the presence of protein aggregates in the cytoplasm of epithelial cells and associated epithelial infolding, and detachment of the smooth muscle cell layer. Epithelial layering and mitotic activity might well be correlated with squamous cell metaplasia, commonly resulting from the exposure of the gland to high estrogen and estrogen-related compounds such as diethylstilbestrol (Risbridger et al, 2001) and attributed to the presence of ERα in the epithelial cells (Chen et al, 2012). Given this piece of evidence, we cannot rule out the idea that the enhanced epithelial cell apoptosis observed immediately after the beginning of the treatment is an intrinsic part of the squamous cell metaplasia. It is interesting to note, though, that inhibition of apoptosis in the BIM knock out mouse resulted in squamous metaplasia of the developing mammary gland duct (Mailleux et al, 2007). It will be interesting to investigate other aspects of this phenomenon, such as the expression of cytokeratin 10 and if it persists at longer periods as

the anti-androgen effect of estrogen takes place. The transient nature of this transition is suggested by the fact that it was not evident when high estrogen was combined with androgen deprivation (Cas+E2 group).

Another interesting finding was the presence of protein aggregates in the cytoplasm of epithelial cells. This is a novel finding and apparently results from a sudden shift in the differentiation program promoted by high estrogen perhaps via ERα. As a matter of fact, microarray analysis (see accompanying manuscript) showed that the expression of the probasin gene was not inhibited by high estrogen treatment, at least within the present experimental conditions. It is possible then, that the protein aggregates correspond to morphological evidence for a unfolded protein response associated with an endoplasmic reticulum stress response in particular its association with the induction of apoptosis (Xu et al, 2005; Woehlbier and Hetz, 2011). This possibility is currently under investigation in our laboratory.

Finally, the combination of androgen deprivation and 17β-estradiol high dose showed aspects of each treatment, but not the epithelial layering typical of squamous cell metaplasia. It is an indication that the observation of apoptotic and mitotic cells might not be intrinsic to the differentiation transition. This group also showed epithelial desquamation typical of the Cas group.

The biochemical analysis of the AKT/PTEN signaling pathways performed here revealed that this pathway is apparently not affected by any of the treatments at the most upstream factors such as AKT and PTEN themselves, but revealed a marked reduction in the phosphorylation state of 4EBP. In the context of the androgen deprivation and high dose 17β-estadiol exposure, 4E-BP activity is highly important because: (1) hypophosphorylated 4E-BP sequesters eIF4E through its $Y(X)_4L\Phi$ motif (which mediates the interaction between eIF4G and eIF4E) and disrupts the eIF4F complex (eIF4G-eIF4E-eIF4A), which is essential for the translation of weak mRNAs containing long and highly structured 5´-UTR, due to eIF4A intrinsic helicase function (Moerke et al, 2007); and (2) the majority of the growth- and proliferation-related proteins have long and structured 5´-UTR (Lodish 1976; Pickering & Willis 2005), so establishing a mechanism to both direct the translation to selected classes of proteins and block proliferation at the level of protein synthesis.

In conclusion, androgen deprivation by castration and high dose 17β-estradiol exposure seems to result in quite different responses in the prostate gland. It became clear that the sudden

fall in androgen levels obtained by castration promotes a series of coordinated events related to inhibition of cell proliferation, inhibition of protein synthesis, and induction of apoptosis. This latter might be indirectly dependent on falling androgen, as suggested by the use of tissue recombinants using epithelial cells lacking the functional AR (Kurita et al, 2001). Androgen deprivation also involved desquamation of groups of epithelial cells, which contribute to the progressive reduction in the number of cells in the gland after castration. On the other hand, estrogen exposure promotes immediate and local effects in the epithelium, including epithelial cell apoptosis. It seems that the presence of androgen is necessary for the squamous metaplasia, since this was much less evident in the Cas+E2 group, suggesting that proliferation and cell death might be distinguished and perhaps independent from the differentiation transition observed in the E2 group. Taken altogether, these results allow for the conclusion that castration and high dose 17β-estradiol and even their combination result in quite distinct changes involving the regulation of cell proliferation, cell death and cell differentiation.

Though the three treatments result in weight loss and regressive changes, they might result in the selection of quite different cell populations and influence the time for relapse of the androgen independent prostate cancer and possibly affect its aggressiveness.

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Figures

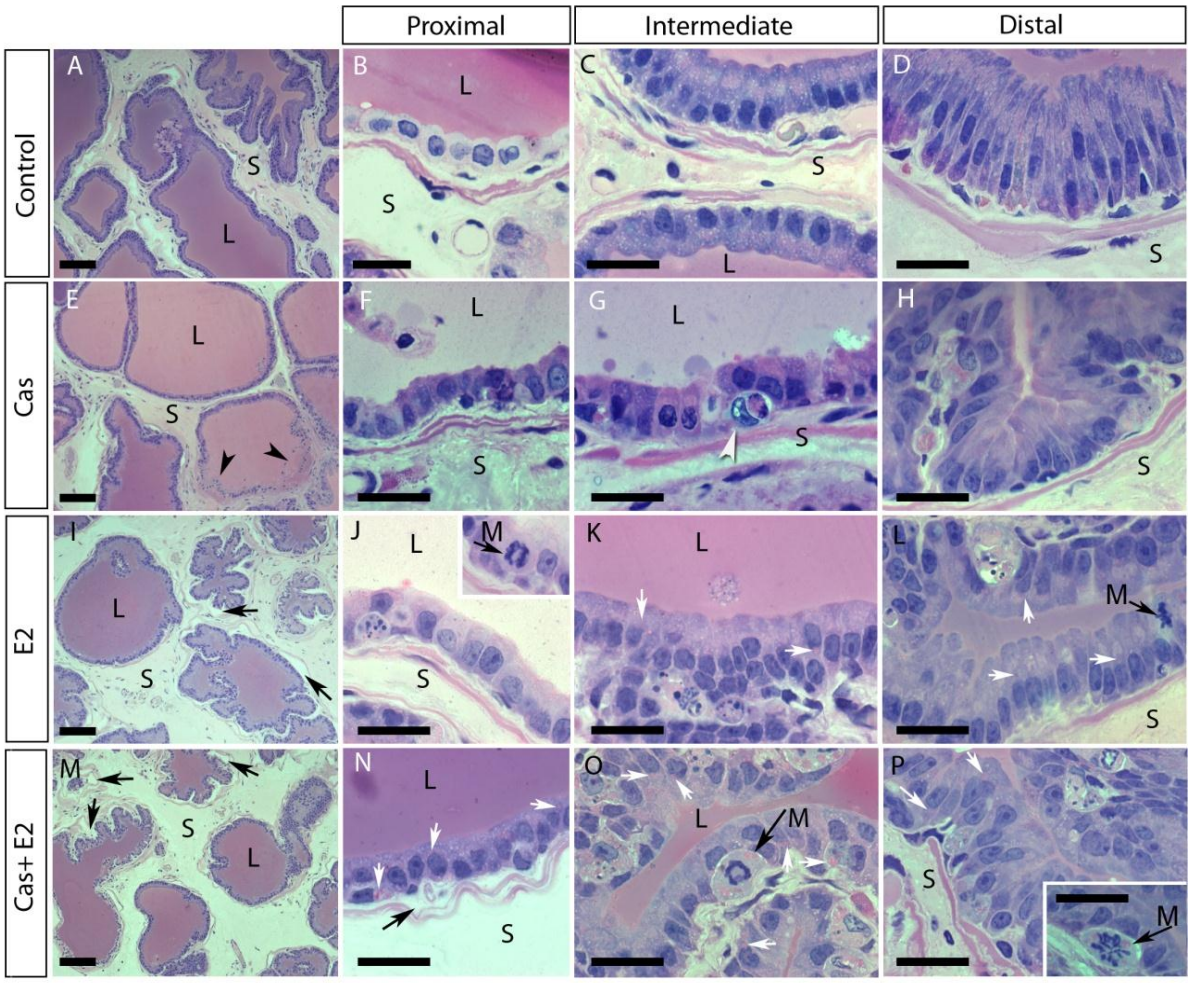

Figure 1. Histological aspects of the rat ventral prostate in the control (**A-D**), Cas (**E-H**), E2 (**I-L**) and Cas+E2 experimental groups (**M-P**) after hematoxylin and eosin staining. Figures **A**, **E**, **I** and **M** are general views depicting the arrangement of the epithelial structures and their lumens (**L**), and the stroma (**S**). Epithelial cell desquamation was observed in the Cas groups (black arrowheads in **E**). The black arrows in **I** and **M** point to detachment of the smooth muscle layers from the epithelial structures which showed a number of infolds in the same regions. Epithelial infolds in the control (**A**), are followed by the smooth muscle cell layer. Details of the proximal (**B**, **F**, **J**, and **N**), intermediate (**C**, **G**, **K** and **O**) and distal ductal regions (**D**, **H**, **L** and **P**) are given. The proximal region in the control group (**B**) shows short cubic cells with a pale stained cytoplasm, large and round nuclei. Multiple smooth muscle cell layers, fibroblast and blood vessels are easily distinguished. The epithelium in the intermediate region (**C**) is populated by tall

cylindrical cells. The stroma is dense in smooth muscle cells, fibroblasts and immune system cells such as mast cells and macrophages. The epithelium in the distal ductal region bears a number of epithelial infolds, the epithelial cells are taller and more elongated than in the intermediate regions, showing similar aspects of secretory activity. Castration (Cas group) resulted in shorter and more intensely stained cells in the three ductal regions (**F-H**), apoptotic cells were frequent and usually appeared phagosomes inside intraepithelial macrophages (white arrowhead in **G**). Desquamated cells were usual in the proximal region. The stroma appeared slightly denser than in the control group. Signs of apocrine secretion were common in the apical region of cells in the intermediate region (**G**). High dose 17β-estradiol (E2 group) resulted in the presence of apoptotic cells along with mitotic cells (M→) in the three ductal regions (**J-L**). The shrinkage observed in the castrated group was not evident, but cells in the intermediated region showed multi-layering. Another interesting observation was the presence o protein aggregates in the cytoplasm of cells in the intermediate and distal regions (**K** and **L**; small white arrows). The histological aspects of the rat ventral prostate in the Cas+E2 group (**N-P**) reproduces most of the findings observed in the Cas and E2 groups, including epithelium desquamation, epithelial infolding and smooth muscle cell detachment (small black arrows), cell shrinkage, apoptotic and mitotic cells in the epithelial layer and protein aggregates inside the epithelial cells (small white arrows). Figures **A**, **E**, **I** and **M**, scale bar = 100 μm; Figures **B**-**D**, **F**-**H**, **J**-**L** and **N**-**P**, insets in figures **J** and **P**, scale bar = 20 μm.

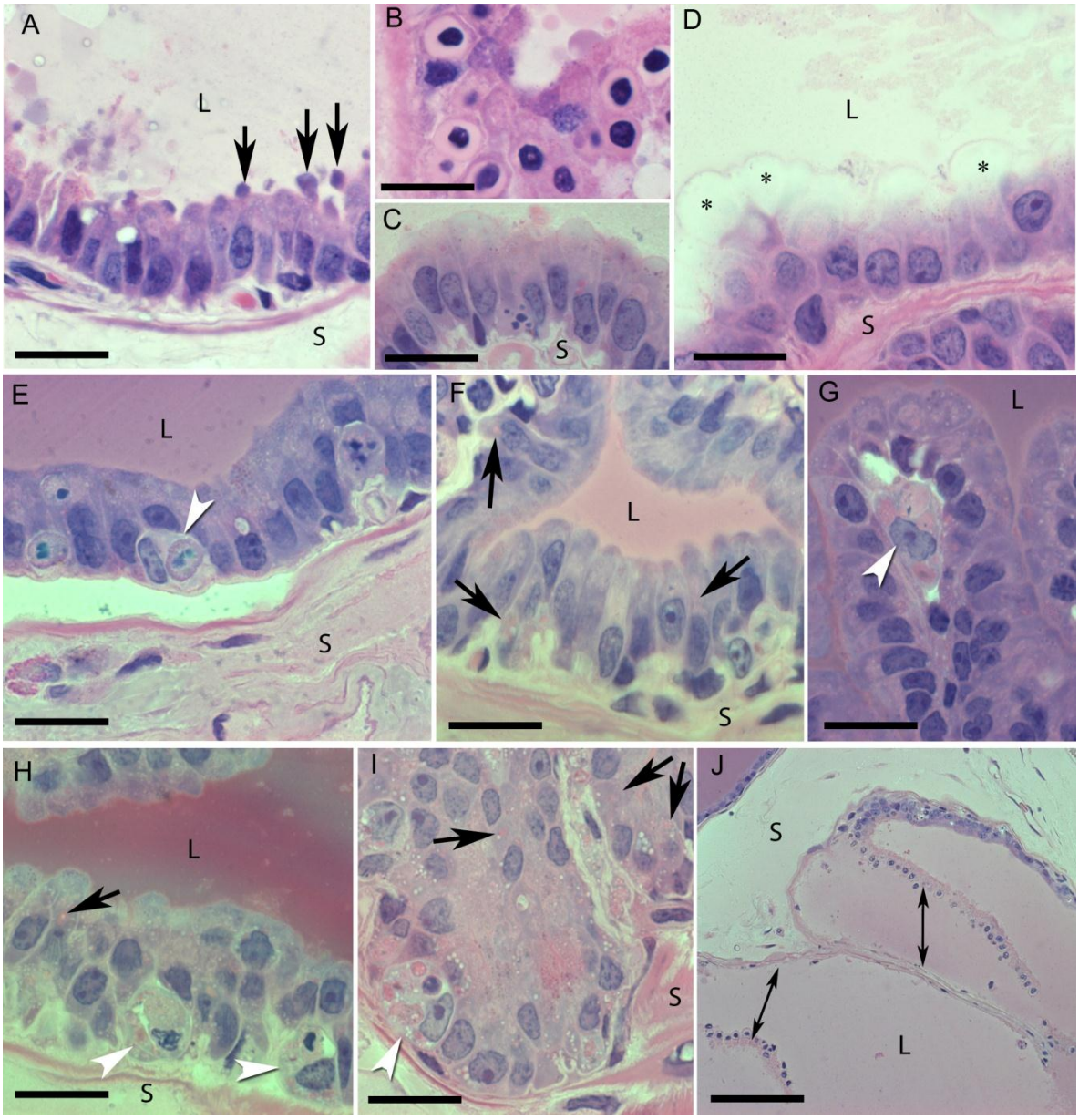

Figure 2. Details of the histological aspects observed in the Cas (**A-D**), E2 (**E-G**) and Cas+E2 group (**H-J**). Castration (Cas group) resulted in shrinkage of the epithelial cells, which showed varied aspect of cell disfunction, such nuclear compaction and apparent homogeneous chromatin, as large vacuoles and aspects of apocrine secretion (black arrows) (**A**). Groups of detached cells found in the lumen showed aspects of cell disintegration such as picnotic nuclei, cell rounding and a homogeneous clear halo around the cell nucleus (**B**). L = lumen; S = Stroma. Aspects of swelling of the apical cytoplasm were commonly found after castration (**C** and **D**; asterisks in **D**). Treatment with high dose 17β-estradiol (E2 group) promoted epithelial cell death, but also promoted unique changes, such as detachment of the smooth muscle cells from the adjacent

epithelial layer (**E**), protein aggregates inside the epithelial cells (**F**; arrows), and an apparently increased phagocytic activity of intraepithelial macrophages (arrowheads in **E** and **G**). The combined treatment of androgen deprivation by castration and high dose17β-estradiol (Cas+E2 group) promoted changes that were observed in either of the individual treatments (H-J). It resulted in various aspects of epithelial cell disfunction, such as layering, the formation of protein aggregates in the cytoplasm (arrows in H and I), increased macrophage phagocytic activity towards dead epithelial cells (arrowheads in H and I), and epithelial cell desquamation, this later being typical from the ventral prostate of castrated rats (J). Figures **A**-**I**, scale bar = 20 μm; Figure **J**, scale bar = 100 μm.

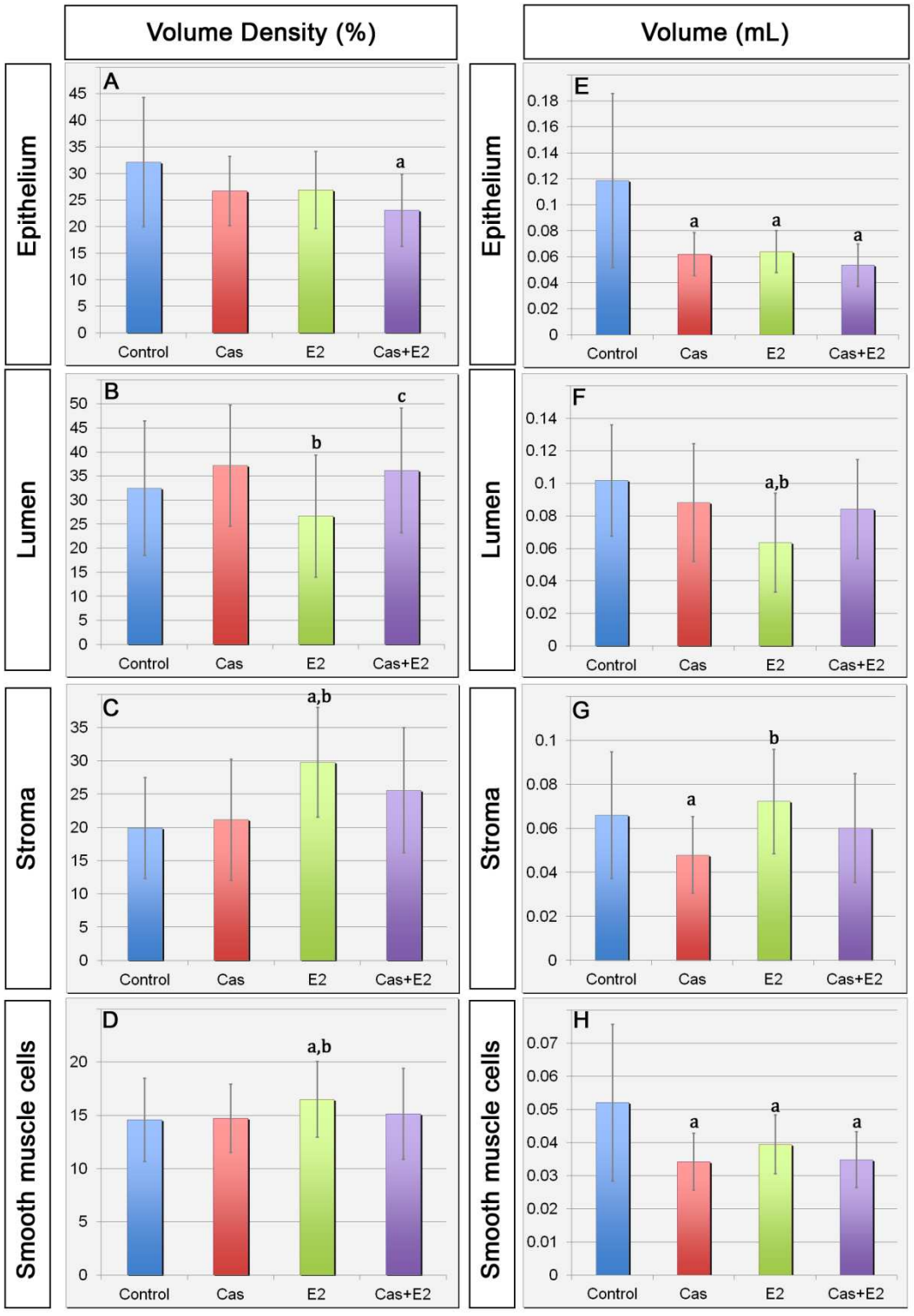

Figure 3. Volume density (Vv%) and volume (mL) of the epithelium, lumen, stroma and smooth muscle cells in the control rat ventral prostate and the changes promoted by castration (Cas group); high dose 17β-estradiol (E2 group), and their combination (Cas+E2 group). Little effect

was observed in the Vv% of the different gland compartments three days after the beginning of the treatment. The results indicated a reduction in the epithelium Vv% in the Cas+E2 group, reduction of the lumen Vv% that were counter balanced by increases in the stroma and smooth muscle cells Vv% in the E2 group. On the other hand, a significant 50% reduction of the epithelial volume and a smaller but significant redution in the volume of the smooth muscle cells were found in the three experimental groups. The luminal volume appeared reduced in the E2 group. The stroma showed a significant reduction in the Cas group. Results are shown as the mean ± standard deviation of the mean. The superscripted letters indicate differences found after ANOVA followed by Tukey's *post hoc* multicomparison test ($p<0.05$). [a]Different from the control group; [b]Different from the Cas group; [c]Different from the E2 group.

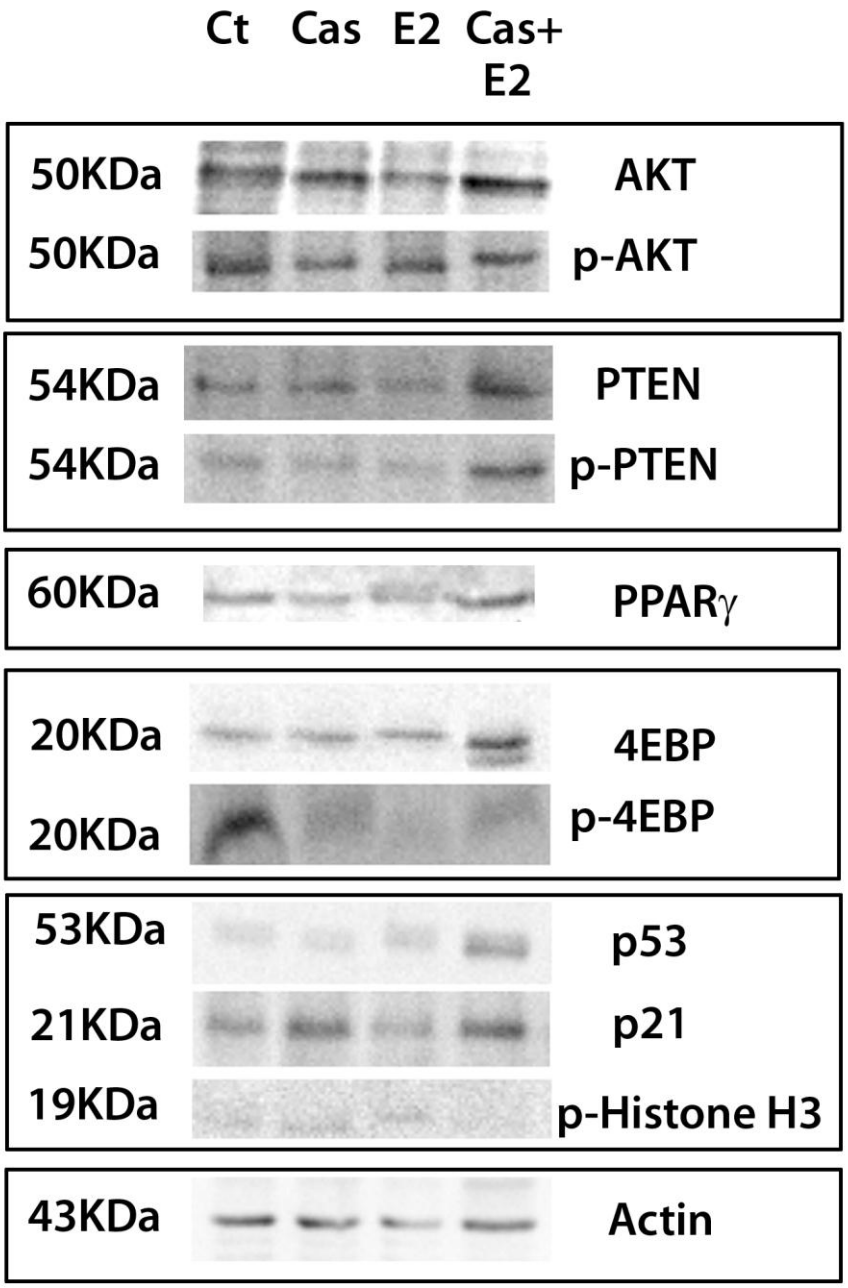

Figure 4. Representative Western blotting results obtained for AKT, PTEN, 4EBP and their phosphorylated forms, PPARγ, p53, p21 and phospho-histones H3 in rat ventral prostate of control animals and in the three experimental groups employed in this work. Total actin was used as a loading control. Densitometry of the resulting bands was normalized by the total actin content in each blotting and the results showed no significant variation in the band intensity, except for p-4EBP (see also Figure 5 for the quantitative data).

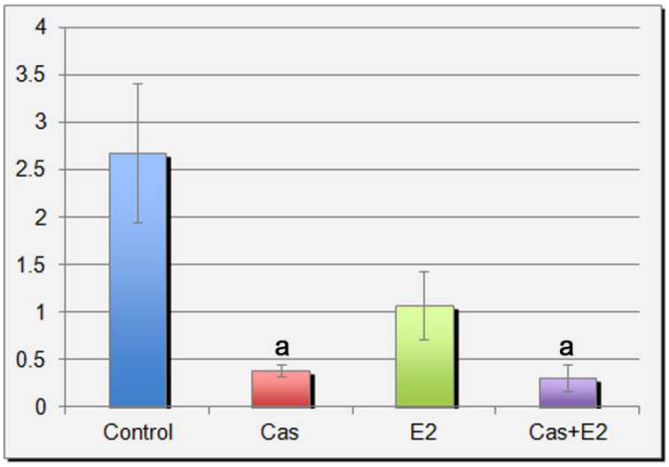

Figure 5. Quantitative data obtained for the p-4EBP/4EBP ratio after Western blotting (see representative images in Figure 4) in the control and experimental groups. Each treatment promoted reduced p-4EBP/4EBP ratios, which were found significant in the Cas and Cas+E2 groups. ANOVA followed by Tukey's *post hoc* multicomparison test ($p<0.05$). [a]Different from the control group (n=3).

Identification of candidate transcription factors for the regulation of rat ventral prostate gland response to androgen deprivation and high dose 17β-estradiol administration

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ABSTRACT

Androgens are key regulators of prostate physiology and exert their effects through the androgen receptor (AR). AR function in transcription regulation is better characterized as an enhancer and is modulated by hundreds of co-activators and co-repressors co-opted to activate or repress a large number of genes. Estrogen acts not only locally in the prostate gland via estrogen receptors, but also as a strong systemic anti-androgen. This work was undertaken to identify candidate transcription factors (TF) working in concert with AR to regulate gene expression after androgen deprivation (Cas), high dose 17β-estradiol (E2) and a combination of both (Cas+E2). We approached this by identifying gene expression profile, gene ontologies and term enrichment pertaining to treatment-exclusive and shared gene subgroups and identifying TF within the regulatory pathways found. We have also performed a search for putative TF binding sites in the proximal promoter of the 10 most up- or down-regulated genes in each experimental group. The results revealed forty-one TF and transcription regulators recovered by both approaches. Among them, Evi-1, NF-Y, HNF-4, Elk-1, GATA-2, c-Rel, v-Myb and NFκB showed differences with respect to the promoter regions of the regulated genes GAPDH and TBP-7. GATA-1 and GATA-2 were characteristic of the Cas group and NFκB of the E2 group. In conclusion, a series of TF was identified as candidates to regulate gene expression profiles in the rat ventral prostate gland response to androgen deprivation and/or high dose estrogen.

INTRODUCTION

Androgens are required for prostate development, growth and physiology. Their action in the gland starts with epithelial budding towards the urogenital sinus mesenchyme during embryogenesis (Timms et al, 1994). The subsequent ductal morphogenesis, canalization, and epithelial differentiation also depend on androgen stimulation and results from a transient increase in testosterone levels occurring around birth (Donjacour et al., 1988). In the normal adult rat prostate gland, androgen receptor (AR) is expressed in both epithelial and stromal cells (Mirosevich et al, 1999; Prins et al, 1992).

There are different studies pointing to the importance of AR in promoting prostate cancer by regulating the growth of prostate cells, hence surgical or chemical castration are first line therapy to promote tumor regression.

The testosterone levels decrease to less than 10% of the control 1-year-old rats as quickly as two hours after castration. The resulting rat ventral prostate weight loss is associated with over 80% of the total cells being lost 10 days after surgery (Isaacs, 1984). A classic peak of prostatic epithelial cell apoptosis occurs three days after castration, and this is much later than the drop in circulating and tissue concentrations of both testosterone and dihydrotestosterone (Kashiwagi et al, 2005). It is also known that events related to the blood supply, blood vessel permeability, and endothelial cell death takes place as earlier as 18-20 hours after castration (Franck-Lissbrant et al, 1998; Shabisgh et al, 1999), suggesting that sequential events precede the epithelial apoptosis peak taking place at 72 hours. We have shown a series of events take place at different time points in the androgen-deprived prostate gland (Vilamaior et al, 2000; Felisbino et al, 2007; Peters et al, 2010), in particular a metalloproteinase-dependent second wave of epithelial apoptosis at day 11 (Bruni-Cardoso et al, 2010).

The AR is a 110 KDa member of the nuclear receptor (NR) superfamily. It binds to androgens, undergo conformational changes, dimerize, and translocate to the cell nucleus (Griekspoor et al, 2007). The AR role in transcription regulation is modulated by several co-activators that interact with one or more regions of the AR molecule. More than 300 proteins that enhance AR activity have been identified (Culig and Santer, 2011). In addition to the co-activators, co-repressor proteins interact with AR in the cis-regulatory regions of the regulated gene (Wang et al, 2007). On the basis of their best recognized primary function, co-activators and co-repressors function as components of the chromatin remodeling complex, as histone modifiers (acetyl-transferases and deacetylases, methyltransferases or demethylases), as components of the

ubiquitination and proteasomal pathways, as components of the sumoylation pathway, as proteins involved in endocytosis, DNA repair system, splicing and RNA metabolism, chaperones and co-chaperones, cytoskeleton proteins, signal integrators, transducers, scaffolds and adaptors, cell cycle or apoptosis regulators (Heemers et al, 2007).

Mapping of AR binding sites in chromosomes 21 and 22 has demonstrated that AR binding takes place at markedly distant from the transcription initiation sites at non-canonical androgen-responsive elements and hierarchical interactions with GATA-2 and Oct1 influences AR activity (Wang et al, 2007).

Besides androgens, estrogen and estrogen-like compounds exert systemic and local effects on the prostate gland. Estrogen anti-androgen effect occurs by blocking the hypothalamic production of gonadotropin-releasing hormone and thereby inhibiting the production of testosterone by the testis. Local effects of estrogens are mediated by ERα and ERβ, which have distinctive tissue distributions (Kuiper et al, 1997). In adult rats, ERβ predominates in the epithelium and ERα is preferentially expressed in stromal cells, including the smooth muscle cells (Weihua et al, 2001). When neonatal rat ventral prostate was treated with testosterone plus estrogen in culture, the growth was retarded and the apoptosis incidence was significantly increased as compared with cultures treated with testosterone alone (Jarred et al, 2000; Taylor et al, 2006). Studies using ERα knockout (ERαKO) mouse prostates have consistently shown that ERα is involved in the prostatic branching morphogenesis and is required for normal prostate development (Chen et al, 2009) and that ERβ regulates prostate growth and hyperplasia by binding both 17β-estradiol and the testosterone metabolite 3betaAdiol (Weihua et al, 2001, 2002).

Rats and mice exposed to estrogen hormone in the perinatal or neonatal period (estrogen imprint) exhibit reduced responsiveness to androgens at puberty and adulthood, frequent inflammatory infiltrates, and increased incidence of epithelial dysplasia at an early age in the prostate gland (Prins et al, 1997; Prins et al, 2001a; Augusto et al, 2010; 2011). In contrast, exposure of the adult prostate to estrogens leads to squamous metaplasia (SQM). SQM is associated with a stratified epithelial layer, cytokeratin 10 expression, and enhanced proliferation of basal cells (Risbridger et al, 2001). The SQM seems to be associated with ERα, because the ERα knockout mouse (pes-ERαKO) is resistant to SQM transition in response to diethylstilbestrol, an ER agonist (Chen et al, 2012).

Previously, we have found differences in the kinetics of epithelial cell apoptosis in the rat ventral prostate by using different androgen/estrogen ratios (Garcia-Florez et al, 2005). This is particularly important because either castration or estrogen might be used as surgical or chemical castration, respectively, and even though they result in prostate regression, there could exist characteristics exclusive to either treatment.

There are increasing evidences demonstrating that additional transcription factors (TF) are necessary for modulating and/or consolidating androgen and estrogen roles via AR and ER in prostate physiology. We have then hypothesized that additional TF could be co-opted for coordinating gene expression that would contribute not only to epithelial cell death but also to an adaptation of the organ to varying hormonal conditions. To pursue this idea, we have performed gene expression profiling using DNA microarrays to identify TF associated with androgen deprivation by surgical castration (Cas group) (low androgen, low estrogen), high dose 17β-estradiol exposure (E2 group) (falling androgen level and high estrogen) and a combination of both (Cas + E2 group) (low androgen, high estrogen). In a first approach, we have identified genes showing differential expression in each experimental group as compared to the controls, arranged them into enrichment terms and ontologies, and identified TF in the resulting regulatory gene networks. In a second approach, we examined the sequence of the 3,000 bp proximal promoter of the genes most differentially expressed for the presence of putative transcription factor binding sites and determined their relative abundance with respect to the corresponding regions in two internal control genes (i.e. not regulated by each treatment). The results revealed a series of TF that appears to be relevant for the fine tuning of prostate response to androgen and estrogens and might contribute to the understanding of prostate physiology under androgen-deprivation, a condition commonly achieved by surgical and/or chemical castration of prostate cancer patients, and for the identification of potential targets for combined drug treatment.

MATERIAL AND METHODS

Animal treatments

Twelve 21-day-old male Wistar rats were obtained from the Multidisciplinary Center for Biological Research (CEMIB), State University of Campinas. The animals were kept under normal light conditions (12 hours cycle) and received filtered tap water and Purina rodent chow *ad libitum*.

At the 90th day after birth, the rats were divided in four groups (n=3) and assigned to different treatment groups. Animals in the first group were castrated (Cas group; low androgen, low estrogen) by orchiectomy via scrotal incision under ketamine (150 mg/Kg body weight) and xylazin (10 mg/kg body weight) anesthesia. Animals in the second group received one high dose 17β-estradiol (E2 group; high estrogen) diluted in corn oil (25 mg/Kg body weight). The third group received a combination of both treatments (Cas+E2 group; low androgen, high estrogen) (combined orchiectomy and one high dose 17β-estradiol). In the control group (Ct; normal androgen, low estrogen) animals received only the vehicle (Garcia-Florez et al, 2005). Three days after the treatments, the rats were killed by anesthetic overdose, and the ventral prostate was dissected out for the analysis.

RNA extraction

Ventral prostates were dissected under RNAse free conditions. Thirty mg of the tissue was used for total RNA extraction. Subsequently, the tissue fragments were extracted using Illustra RNAspin Mini kits (GE Healthcare, Germany) according to the manufacturer's instructions. RNA purity was analyzed by the absorbance ratio 260/280 (values higher than 1.8) and by electrophoresis on 1.2% denaturing agarose gel. The RNA concentration in each sample was determined in an Ultraspec 2100pro spectrophotometer (Amersham Biosciences).

Microarray hybridization

Whole transcript microarrays (GeneChip Rat Gene 1.0 ST Array) purchased from Affymetrix were used for gene expression analysis. Microarray probes were synthesized from 500 ng of total RNA using WT Expression kit (Ambion, Austin TX, USA) according to the manufacturer's instructions. Single strand cDNA was synthesized containing a T7 promoter sequence and the second-strand cDNA was synthesized by DNA polymerase in the presence of RNase H.

The antisense cRNA was synthesized and amplified by *in vitro* transcription (IVT) of the second-strand cDNA template using T7RNA polymerase. The cRNA obtained was purified to improve the stability of the cRNA. From 10 μg of purified cRNA the sense-strand cDNA (ss-cDNA) was synthesized by reverse transcription using random primers and the ss-cDNA

contained dUTP at a fixed ratio relative to dTTP. Then, the cRNA was degraded by RNase H and the ss-cDNA was purified.

In the second part of the protocol, 5.5 ng ss-cDNA in a 31.2 µL volume were nicked and labeled using the GeneChip WT Terminal Labeling Kit (Affymetrix) according to the manufacturer's instructions. The efficiency of the labeling procedure was controlled by a protocol that prevents hybridizing poorly labeled targets onto the probe array. The addition of biotin residues was checked in a gel-shift assay using 4% agarose gel in TBE buffer and monitoring for the presence of fragments with 200 bp or less.

For the hybridization, wash and staining steps, the GeneChip Hybridization, Wash and Stain Kit (Affymetrix) was used. The 80 µL of the fragmented and labeled ss-cDNA solution was loaded on the gene chip probe array and incubated at 45ºC, 60 rpm for 18 hours. The hybridized chip was set in the fluidic station to stain and wash (GeneChip Fluidics Station 450; Affymetrix), and scanned using a Affymetrix GeneChip Operating Software v1.3 program. The data was summarized and normalized by Expression Console software (Affymetrix) and the results were analyzed by bioinformatics.

Microarray results analysis

The expression profile was done for biological replicas from treated and control groups. Fold-change was determined using R script and by the Aff and Limma library; an associated p-value was used to select genes for subsequent analysis. Genes with fold-change >1.5 or < -1.5 and p-value <0.01 were employed for subsequent analyses. The ID of each gene was found using the Affymetrix annotation files.

Genes appearing to be exclusive or shared (intersections) by the experimental groups were determined using the script PERL and presented as Venn diagrams. Enrichment term analysis was performed for each of the subgroups (exclusive and shared genes), comparing their frequencies with the rat transcriptome, using the IPA Ingenuity software. Gene ontologies were determined using the DAVID on line platform for the same subgroups appearing in the Venn diagram and grouped according to the major category (a) cell proliferation, (b) cell death, (c) immune response, and (d) "others". The number of ontologies in each category was determined and showed as bar graphs.

Transcription factor and transcription regulators in the enrichment terms

Enrichment terms were determined for each subgroup (exclusive and shared genes) using the IPA Ingenuity software. The genes involved in each enrichment term were grouped in regulatory networks. Finally, the transcription factors and transcription regulators enrolled in the regulatory networks were identified and classified as having differential expression in the microarray data or not.

TF binding sites in the proximal promoter region of most regulated genes

After selecting the 10 up- and 10-down-regulated genes exclusive to each treatment and those shared by the three treatments (n=80), we analyzed each gene individually to determined putative TF binding sites in the proximal 3,000 bp promoter region. The sequences were loaded in the Match-1.0 program (www.gene-regulation.com) and the parameters (a) vertebrate matrices and (b) cut off for the false positives and negatives matrix groups were selected to identify the TF binding sequences.

The resulting list was sorted according to the total amount of binding sites in the promoter regions. We also made a comparison of the number of binding sites for the identified TF with those in the proximal promoter region of the GAPDH and TBP-7 genes, which were selected as internal controls, as their mRNA levels were not affected by the treatments, as determined by qRT-PCR.

The TF density (number of binding sites in the promoter region divided by the number of genes) in the regulated genes was compared with the TF density in the promoter region of the internal control genes, as a measure for eliminating those with a wide distribution in the genome. Finally, we identified the TF that were exclusive (or enriched) to each experimental group.

RESULTS

Microarray data

Microarray analysis showed the gene expression profile of the treated groups as compared with control animals. A 1.5-fold variation and a $p<0.001$ value were considered for selection of the genes with differential expression. This resulted in a total of 2,693 genes showing differential expression as compared to the controls. One fifth (21.5%) of these genes (580 genes) were shared by the three experimental conditions employed in this work (226 up and 354 down), as shown in the Venn diagrams from Figure 1. In general, the genes shared by the three experimental groups (shared genes) had similar fold-variation i.e. appeared as up- or down-regulated and presented approximately the same change-variation in each group.

Besides sharing these differentially expressed genes, the Cas group showed the largest number of differentially expressed genes (2,046 genes), while the E2 group showed 830 genes, and only 132 exclusive genes. The combined treatment (Cas+E2 group) showed an intermediate number of differentially expressed genes (1,856 in total) with 473 exclusive genes.

It became clear that androgen deprivation (Cas group) resulted in more exclusive genes that were up-regulated than those that were down-regulated, suggesting that the changes achieved by androgen deprivation require an active process of gene expression to coordinate the modifications associated with gland regression and tissue remodeling. In contrast, more genes were down-regulated in response to E2 treatment or the combination of Cas+E2. Surprisingly, the gene coding for probasin, a product of the secretory activity of the rat ventral prostate, was not down-regulated in the E2 group, whilst it showed a 101-fold decreased expression in response to androgen deprivation (Cas group) and 39-fold decrease after the combined treatment (Cas+E2 group).

Gene ontologies

The regulated genes in each subgroup were organized according to their ontologies. Different and important functional groups were found in this analysis. The number of ontologies containing growth and proliferation, cell death and apoptosis, and immune response corresponded to a large fraction of the total.

The 580 genes shared by the three experimental groups were organized in 224 ontologies. The number of ontologies involving growth and proliferation, cell death and apoptosis, immune response and others (Figure 2) showed that in spite of sharing a number of genes with the other

experimental groups, the Cas group exhibited 124 ontologies related to the selected process that were exclusive to this hormonal situation.

Those genes in the E2 group were organized in 36 ontologies with no representatives in the cell death and apoptosis and in the immune response classifications. The number of gene ontologies related to growth and proliferation was similar to that found for the Cas group. The Cas+ E_2 group showed 64 ontologies (Figure 2). In this group, the number of ontologies related to immune response was the highest for the individual groups.

The intersection between the Cas and Cas+E_2 groups showed four different classifications. This group is interesting because it unveils that beyond the 224 ontologies found at the intersection between the three experimental groups, the two treatments share additional 270 ontologies involving the four global classes (growth and proliferation classification, cell death, immune response and others).

TF in the enrichment terms

Enrichment terms were observed in the Cas (37 terms) and E2 (5 terms) groups and in the intersections between the three experimental groups (common genes) (90 terms) and in the intersection of the Cas and Cas + E_2 groups (88 terms) (Figure 3).

After the identification of the enrichment terms, we grouped all genes in each subgroup and used the IPA Ingenuity software to identify gene networks connecting the different molecules. Then, the transcription regulation related genes showing in those networks for each of the exclusive or shared genes were isolated (Supplemental Table 1) and those which showed differential expression in the microarray data for each of the experimental groups were listed (Table 1). From the present analysis it became clear that more TF were up-regulated than down-regulated in each experimental condition.

TF in the promoter region of the 10 most up- or down-regulated genes

From the microarray analysis, we selected the ten most up- or down-regulated genes as compared to the controls, exclusive for each experimental group and those shared by the three treatments (Table 2).

For each of these genes, we did a search for transcription binding sites in the proximal 3,000 bp promoter region. This analysis was extended to the GAPDH and TBP-7 genes, which

showed no differential expression in the different experimental groups, as determined by qRT-PCR, as a measure of their concentration in the promoter of the regulated genes.

We found a series of putative TF binding sites that were absent from the promoter region of the internal control genes (Figure 4A). Most of them showed low density. Figure 4B shows a comparison between the density of TF binding sites found in the promoter region of the regulated genes and their density in the promoter region of the internal controls. The transcription factors CDP CR1, Evi-1, CCAT box, Brn-2, Freac-7, NF-Y, HNF-4, FOXD3, Elk-1, GATA-2, SOX-9, CP2, CHOP-C/EBPalpha, HFH-3, Pax-6, v-Maf, c-Rel, GATA-3, v-Myb, Barbie Box, FOXJ2, Gfi-1, Hand1/E47, Sox-5, Oct-1, NFκB, GATA-x, COMP1 showed binding site densities in the promoter of the regulated genes that were equal or higher than in the promoter region of the non-regulated genes (GAPDH and TBP-7).

Next, we paired these transcription factors to examine the contributions from genes regulated in each of the experimental conditions tested in this work, using a logarithmic scale (Table 3). The results revealed that Evi-1 is highly frequent in the differentially expressed genes in the intersection of the three treatments (shared genes). GATA-1 and GATA-3 are highly frequent in the Cas group and NFκB was less frequent in the intersection of the three experimental groups, while highly frequent in the E2 group. In addition to the logarithmic scale, we also considered the transcription factors that showed 2-fold higher frequencies (as compared to one another) and found GATA-2 to be highly frequent in the Cas group and HNF-1 in the subgroup of genes in the intersection of the three experimental groups (Table 4).

Finally, we compared the two lists to identify the TF highlighted by both approaches. Forty-one transcription factors were selected through both approaches. Those which showed highly frequent putative binding sites in the promoter regions of the regulated genes were Evi-1, NF-Y, HNF-4, Elk-1, GATA-2, c-Rel, v-Myb and NFκB. Those showing high differences among the experimental groups were GATA-1 and GATA-2 in the Cas group, and NFκB in the E2 group. HNF-1 was also identified by both approaches but was characteristic of the intersection between the three experimental groups.

DISCUSSION

By comparing the genes expression profiles in the rat ventral prostate under different hormonal conditions (androgen deprivation, high 17β-estradiol dose and their combination) we

could identify differentially expressed genes, different term enrichment, differences in ontology organization and a series of TF which are either differentially expressed in each experimental group or shared between them and that represent candidates to contribute to AR and ERs in the regulation of gene expression in response to varying ratios of the androgen and estrogen levels.

When the ventral prostate was subjected to the different hormonal regimen employed here, different kinetics of apoptosis were achieved. The classical peak was found in the Cas group in the third day, but estrogen administration resulted in an immediate increase in apoptosis rate, while the combination of the two showed additional effect, with a higher number of apoptotic cells at day 3, suggesting that different apoptosis pathways were activated by either androgen deprivation or high dose 17β-estradiol (Garcia-Florez et al, 2005).

Previous DNA microarray studies have investigated the differential gene expression in the prostate gland in response to androgen deprivation and reposition to identify androgen-regulated genes. Pang et al. (2002) have found thousands of genes and concentrated on those related to the oxidative stress response, suggesting that variable androgen levels might result in reduced protection against reactive species and this could be at the very center of age-related prostate cancer initiation and progression. Desai et al. (2004) also employed a similar approach and concluded that a particular involvement of immune system cells is triggered in response to androgen deprivation and their conclusion was reinforced by another study employing isolated epithelial cells (Asirvatham et al, 2006).

The present study reveals a large number of genes exclusively expressed in each experimental group and a number of genes, which were shared by the three or each treatment pairs. The Venn diagrams shown in Figure 1 depict the number of genes in each subgroup. It became clear that androgen deprivation contributed with most of the regulated genes, while the E2 group showed the least number of genes differentially expressed. It was interesting to note that genes involved with the secretion activity such as the probasin gene, and genes associated with cell proliferation were not down regulated in the E2 group.

By examining the enrichment terms, we found that the Cas group show the highest number (215 in total, 37 exclusive) and the E2 group showed only 5 exclusive terms. The notable result from this analysis was though the fact that the Cas+E2 group showed no exclusive enrichment term. To our understanding, it fits well to the morphological analysis we have performed, showing that the ventral prostate in this group depicts aspects from each individual

treatment (see accompanying paper) and to our previous suggestion that the combined treatment shows an addictive effect with regard to the rate of apoptosis, but function eventually through distinct mechanisms (Garcia-Florez et al, 2005).

It is well understood that treatment with androgens increases the AR and coactivator occupancy in the PSA enhancer or promoter regions (Jia et al, 2004; Kang et al, 2004; Louie et al, 2003; Shang et al, 2002; Wang et al, 2002). Confirming that AR has an enhancer function in the regulated genes (Wang et a, 2007), we have found 5 AR binding sites in the promoter region of the ABCC3 gene, and at least two located at -12,529 and -12,572, with great potential for binding activated AR dimers (unpublished results).

Besides AR, other transcription factors respond to androgen in the PSA enhancer and promoter regions, such as HATs, AcH3, RNA polymerase II and phosphorylated RNA polymerase II. The ER is not recruited to these regions of the PSA gene promoter in LNCaP cells (Wang et al, 2005).

Instead of analyzing the differentially expressed genes themselves and ending up with a selected group of genes representing a specific physiological function, we decided to search for TF that could be regulating the organ response and a variable set of functions.

We identified TF genes in regulatory networks established from the enrichment terms and from the analysis of the proximal promoter region of the genes that were regulated by androgen deprivation and estrogen stimulus as shown by the microarray analysis. The comparison of these results unveiled a list of interesting TF candidates (Evi-1, NF-Y, HNF-4, Elk-1, GATA-2, c-Rel, v-Myb and NFκB) that are suggestively co-opted by AR and/or ERs to regulate gene expression to varying levels of androgens and/or 17β-estradiol.

The Evi-1 TF has been found overexpressed in ependymonas. Its inhibition promoted significant impairment of cell proliferation, and appeared as worse prognostic factor in infratentorial ependymonas (but not in supratentorial tumors) (Koos et al, 2011). Evi-1 was identified in the interactions of enrichment terms for both Cas and Cas+E2 treatment groups and also as one of the TF with frequent binding sites in the proximal promoter of the 10-most up- or down-regulated genes. It was interesting to note that Evi-1 binding sites were found in both up- and down-regulated genes in the three experimental groups, suggesting that it is a candidate to cooperate with general transcription factors in the ventral prostate in the response to variations in steroid hormone concentrations.

Another TF uncovered by the two approaches was HNF-4. It is involved in the networks of genes found in enrichment terms at the intersection of the three treatment groups, suggesting its involvement in the regulation of events common to either androgen deprivation or high dose estrogen, which is also indicated by the wide distribution in the promoters of genes regulated in the three experimental groups and also in their intersection. It is worth mentioning, that among the HNF-4 family members, HNF4alpha1 binding sites were concentrated in the promoter region of CFh gene coding for complement factor H (unpublished result). Complement factor H is expressed by the fetal and adult liver, tongue and salivary gland and also by smooth muscle cells. There is also an indication that CFh is expressed by prostate stromal cells (Orr et al, 2011).

NF-Y is known to associate with CCAAT motifs in the promoters of cell cycle regulatory genes and positively regulate their transcription. In a recent study, it was found that cyclin-A promote the binding of NF-Y to CCAAT sequences in the CDC2 promoter, concomitant with the transcription of this gene (Chae et al, 2011). On the other hand, the involvement of NF-Y binding in the promoter region of Bim gene of neurons accommodates the idea of a possible role in regulating cell death (Hughes et al, 2011). The effective role of NF-Y in the regulation of gene expression in the prostate gland needs to be investigated, particularly given is assortment as a TF involved in the regulation of enrichment terms in the Cas and Cas+E2 groups, and those found for the genes shared by the three experimental groups.

Elk-1 revealed a relationship with PI3K/AKT pathway, for promoting cell proliferation. The PI3K/AKT pathway via the MAPK/ERK1/2 activates Elk-1 in lung carcinoma cells to induce cell proliferation (Zhang et al, 2006). EGF also induces the transactivation of Elk-1 in hepatocellular carcinoma cells (Pusl et al, 2002). It was interesting to find that this TF was present only in networks obtained from enrichment terms in the E2 group and might somehow be involved with the maintenance of the proliferative state of a fraction of cells in this group. Elk-1 is suggestively involved with the squamous cell metaplasia (see accompanying paper) even though it is evenly distributed in the proximal promoter region of most regulated genes in the other treatment groups, with a notable concentration in the MRPL41 gene which was down regulated in the Cas+E2 group. MRPL41 nuclear gene codes a mammalian mitochondrial ribosomal protein involved in ribosomal structure and protein synthesis inside the mitochondria (Goldschmidt -Reisin et al, 1998) but also correlates with suppression of cell cycle progression and apoptosis induction by interacting with p53, p21 and p27 (Yoo et al, 2005; Kim et al, 2006)

and by binding Bcl-2 (Chintharlapalli et al, 2005), respectively. This putative pathway is important by connecting estrogen stimulation, cell proliferation, and cell death and metabolism, which is particularly interesting in the prostate due to the active involvement of the luminal epithelial cells in androgen-stimulated gland in pumping citrate, a major substrate of the tricarboxylic acid, to the lumen.

GATA TF was present in association with androgen responsive elements (ARE) in the far upstream PSA enhancer (Schuur et al, 1996; Cleutjens et al, 1997a; Pang et al, 1997; Zhang et al, 1997). Studies on the GATA gene family revealed that GATA-2 is expressed in LNCaP cells but not in PC3 cells, which do not express the AR. In contrast, the estrogen-responsive human breast cancer cells (MCF-7) express GATA-3 and low levels of GATA-2 and GATA-6 (Perez-Stable et al, 2000). Thus, it appears that there is a correlation between the estrogen receptor and GATA-3 in some estrogen responsive breast cancers (Hoch et al, 1999). In the present study, GATA-2 was identified by both approaches as a candidate TF. GATA-1 and GATA-2 were characteristically more frequent in the promoter regions of genes differentially modulated in the Cas group. Curiously, most of the binding sites contributing to the concentration of GATA-1 and GATA-2 in the promoter regions of the modulated genes were found in the OCM gene (oncomodulin-2; *OCM2* or *LOC4951* in humans), which was one of the ten genes most down-regulated by androgen deprivation. Oncomodulin is an EF-hand calcium binding protein found in embryonic cells, in the placenta and in many tumors. In the adult, it is expressed in the outer hair cells of the organ of Corti (Sakaguchi et al, 1998). Curiously, a denser array of binding sites for FOXD3, FOXJ2, Freac-7, GATA-3, HFH-3, HNF-3beta, HNF-4 and Oct-1 was found in the proximal promoter of the OCM gene (result not shown). While this dense array of transcription factor binding sites might be related to the repression of a gene highly expressed during development and in tumors, it is not easy to understand why this gene is down regulated by androgen deprivation. It will be interesting to search for the cell types expressing OCM in the prostate gland during normal androgen stimulation and try to determine its function in the organ.

Interestingly, GATA-2 was found as a *cis*-active element in the promoter region of PSA and TMPRSS2 genes. In that study, GATA-2 and Oct-1 mRNA knock down promoted decreased expression of the two genes in response to androgen (Wang et al, 2007).

In our results we found that GATA-2 and Oct-1 showed a high density of two of binding sites in the promoter region of the regulated genes as compared with GAPDH and TBP7 genes.

GATA-2 binding sites appeared in the promoter regions of in genes regulated by castration (Cas group) and as a regulator of the networks obtained for the enrichment terms recovered from intersection between the Cas and Cas+E2 experimental groups, reinforcing its role as an important TF co-opted by AR to regulate the gene expression in the prostate gland.

v-Myb was another transcription factor with high density of binding sites in the promote region of the regulated genes. It was identified in the Cas and Cas+E2 enrichment genes network. V-Myb is frequently overexpressed in ER-α expressing breast cancer cells (Ramsay et al, 2008). Data obtained in studies with the MMTV-PyMT mouse suggested that v-Myb is essential for the development of mammary tumors but was not ultimately essential for their progression (Miao et al, 2011). *In vitro* tests showed that normal epithelial mammary cells NMuMG transduced with Myb presents high proliferation and reduced apoptosis (Zhang et al, 1998). The regulated genes with binding sites in the proximal promoter region showed wide variation. Those with the highest number of binding sites were LOC310926 (cytochrome c oxydase 1), SHeRF2 (putative E3 ubiquitin protein-ligase) (both up-regulated in the Cas+E2 group), and Slc39 (solute carrier family 39 zinc transporter) (up-regulated in E2 group). It has been shown that the anti-estrogen The effect of Fulvestran to hamper zinc accumulation in mammary gland cancer cells was not associated with Zip6 (LIV-1) (another zinc transporter) expression (Lopez and Kelleher, 2010). It seems interesting to compare Slc39 gene product activity with the zinc pumping activity of prostate epithelial cells, similarly to citrate, as above mentioned.

NFκB binding sites were concentrated in the promoter region of genes regulated in the E2 group and appeared as a regulator of the gene networks in the Cas group and in the intersection between the Cas and Cas+E2 treatment groups. NFκB involvement in prostate cancer is extensive. The castration-resistant prostate cancer (CRPCa) exhibits chronic NFκB expression (Suh et al, 2002), NFκB appears to regulate TGFβ-induced epithelial-to-mesenchymal-transition (EMT), which is thought to be a key process during the metastasis of solid tumors (Zhang et al, 2009). Results from *in vitro* experiments suggest that NFκ-B interferes with AR expression, showing that AR is a NFκB target gene.

Different combinations of the NFκB dimers promoted variable effects on AR gene transcription, but overexpression of the major NFκB factor p65/RelA caused increased AR mRNA and protein levels (Jain et al, 2011). In the E2 group, NFkB binding sites concentrated in the down-regulated genes (present in 7 out of the 10 most down-regulated genes against 3 out of

the 10 most up-regulated genes). We also noticed a concentration of NFκB binding sites in the promoter region of the SDR16c5 gene, which appeared up-regulated in the Cas+E2 group. Epidermal retinol dehydrogenase 2, the product of the SDR16c5 gene, is a short chain dehydrogenase, processing retinol to all-trans retinaldehyde, which in turn can be converted to all-trans retinoic acid. It is possible that under estrogen stimulation, the prostate can produce retinoic acid, an important regulator of prostate development and physiology (Prins et al, 2001b; 2002).

c-Rel is another TF identified in the present work. C-Rel is a 69KDa subunit of the NFκ-B family and, as such, it is involved in immune response, inflammation, stress response, embryonic development, growth control and apoptosis (Barkett and Gilmore, 1999). c-Rel was found to be physically associated with AR in both cytoplasm and nucleus of LNCaP cells after R1881 treatment with the AR agonist R1881 and that cRel represses AR transcriptional activity (Mukhopadhyay et al, 2006). Here we observed that c-Rel appeared in the enrichment terms network in the intersection between the Cas and Cas+E2 groups and was one of most frequent TF binding sites in the promoter of regulated genes. This suggests that c-Rel might influence the expression of genes in response to androgen deprivation.

It is also possible that members of the NFκB family represent a molecular switch connecting androgen, estrogen and retinoic acid stimuli to coordinate prostatic epithelial cell proliferation, differentiation and apoptosis, given their role in apoptosis (Barkett and Gilmore, 1999) and constitutive expression in tumors (Suh et al, 2002; Zhang et al, 2009)

Androgen deprivation and estrogen are questionable and controversial therapeutic measures in the treatment of prostate cancer. The major conclusion from this work is that their effects and mechanisms are not similar and point to the necessity of further investigating the physiological response of the prostate gland to androgen-deprivation and anti-androgen therapies for better predicting and perhaps controlling the disease relapse in the form of androgen-independent prostate cancer.

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Figures

Table 1. Transcription regulation related genes indentified by IPA Ingenuity program and showing differential expression in those subgroups containing enrichment terms. The red and green colors highlights the up- or down-regulated genes as compared with control group, respectively.

Shared	Cas	E2	Cas and Cas+E2
AATF	ID2	BRCA2	ANKRD6
JUN	IKZF2	CBX3	ARID5B
MED12	IRF5	SIRT1	BTG2
MYBL1	IRF8	PURA	CITED1
NPM1	IRF9		EGR1
TGIF1	MEF2C		ELF4
TRIM24	SMAD3		FOXK1
ARNTL	SNW1		FOXQ1
CLIP2	STAT1		HCLS1
NFX1	TAF15		HMG20B
RAD54L2	OVOL1		HMGB2
TAF9B	POLD3		KLF6
TCEA3	TAF1B		KLF11
TCEB1	ZEB2		NFKB2
	TCEB1		NPM1
			PTTG1
			STAT6
			VAV1
			ZFP57
			EAF2
			HOXC6
			ID4
			NKX3-1
			TCEB1
			TFDP2
			TMF1

UP

DOWN

Table 2. The ten most up- or down-regulated genes exclusive to each of the experimental groups or shared by three treatments. These genes are involved in different functions (see Appendix 1 for details on the annotated functions for these genes).

Groups	Up-regulated	Down-regulated
Cas	Ska3/ LOC689296/ LOC681325 Aspn /RGD1562462/Slamf9 Ifit1/Kcnd2/Rnase2/ Rpl21	Ocm/ Furin/ Isyna1 LOC287167/Aox3/Dmd/Slc17a9 Tomm7/Tnnt2/Hspb6
E2	Ankrd11/N5/Hamp/Sft2d1 LOC691979/ nucleolin/Tns4 Slc39a1/Dync2h1/LOC290071	Sparc/Slc25a24/Susd2/Lcn2 Ly6c/Pi16/Glrx1/Kcnmb1 C1H6orf35 /Tmem47
Cas+ E2	Sh3rf2/LOC310926/ Macc1/Chi3l1/Krt15/LOC259246 Rnase1l1/Sdr16c5 /Obp3/Prol1	Mrpl41/ Sms/ Defb50/Hectd2 MGC109340/Scnn1g/Me1 V1rd15/Twf1/Pmp22
Shared	RGD1565682/ Cfh/Gnat2 Lrrc34/Mmp12 /Gpnmb /Abcc3/V1rk2/ Blnk /Mmp15	Ugt2b34 /Cntn6 /Cyp2c13 Oas1k/Neto2/Cpa6/Cdh12 Wfdc3/Lrrc37a/ Ldoc1

Table 3. TF binding sites in the promoter regions of the 10-most up- and down-regulated genes with density values falling within different orders of magnitude (log scale) in the selected groups.

	Shared	Cas	E2	Cas+E2
Evi-1	100	92	69	67
GATA-1	59	107	86	73
GATA-3	66	108	52	60
NFκB	8	18	49	18
CHOP-C/EBPalpha	19	22	8	15

Table 4. TF binding sites in the promoter regions of the 10-most up- and down-regulated genes showing 2-fold higher density values among any of the selected groups (exclusive or shared by three).

	Shared	Cas	E2	Cas+E2
Elk-1	37	67	64	75
GATA-2	43	86	53	48
Sox-9	60	57	30	51
CCAAT box	58	50	27	26
HNF-1	51	35	22	30
HFH-3	32	44	33	19
NF-Y	36	33	12	22
NFκB	8	18	49	18

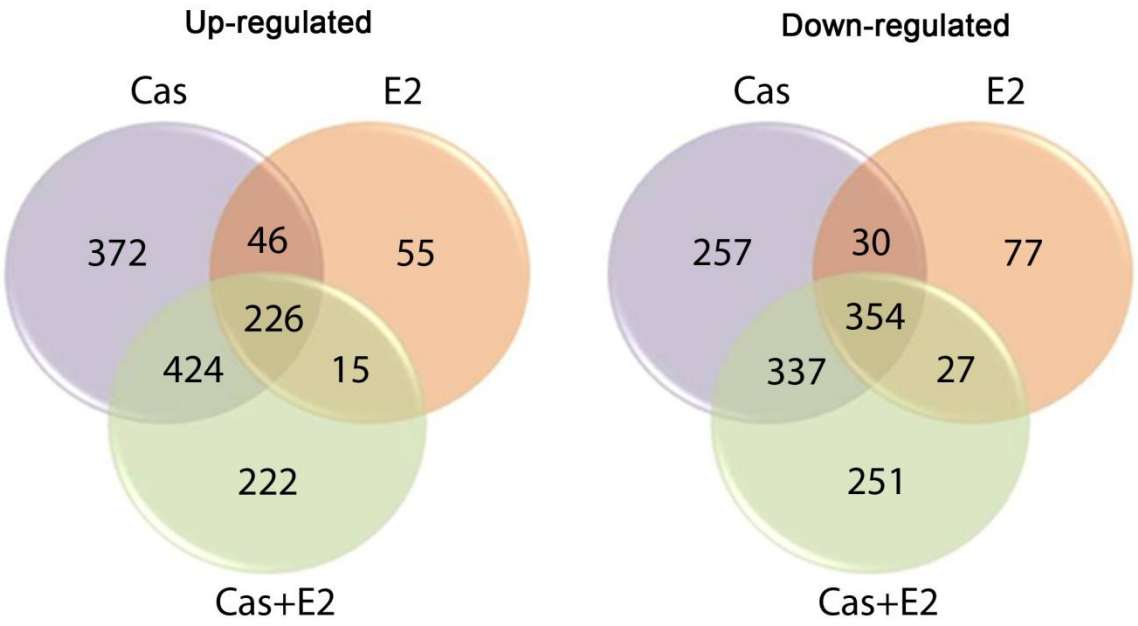

Figure 1. Venn diagrams showing the number of genes differentially expressed in the three experimental groups (Cas; E2 and Cas+E2) as compared with control group, revealing those exclusive to each treatment and those shared by the three or each pair.

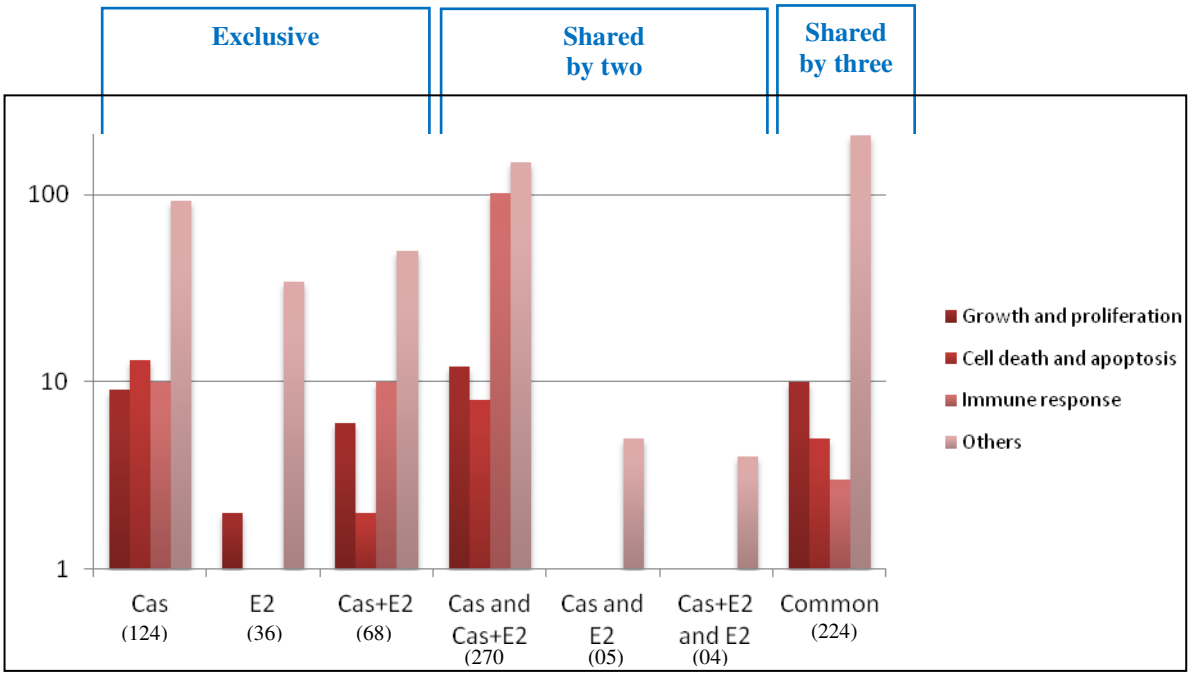

Figure 2. Distribution of the total number of ontologies related in the four global classifications for the genes differentially expressed in the experimental groups (Cas, E2, Cas+E2) and distributed according to their characteristic of being exclusive or shared between the three treatments. The numbers in the parentheses correspond to the total number of ontologies per subgroup.

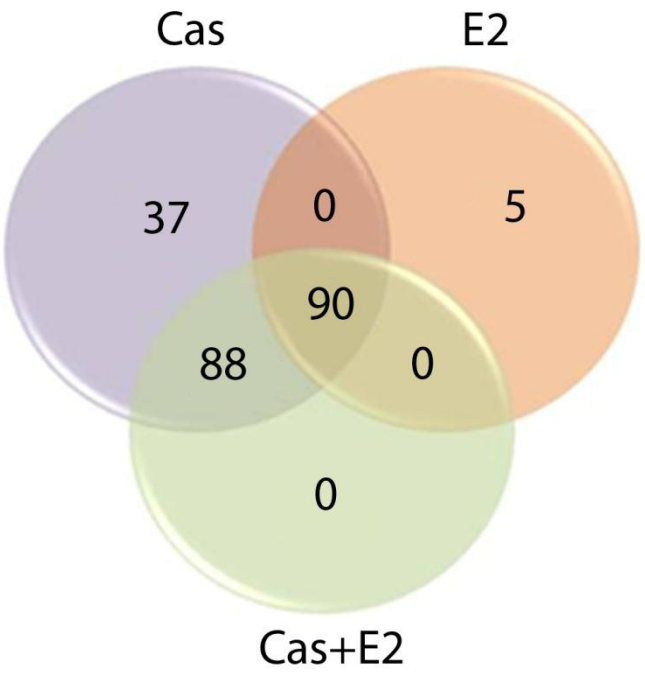

Figure 3. Number of enrichment terms found for the groups of genes exclusive for each or shared by the three experimental groups. The three treatments share 90 enrichment terms. The highest number of enrichment terms was found in the Cas group. The Cas+E2 group showed no exclusive enrichment term.

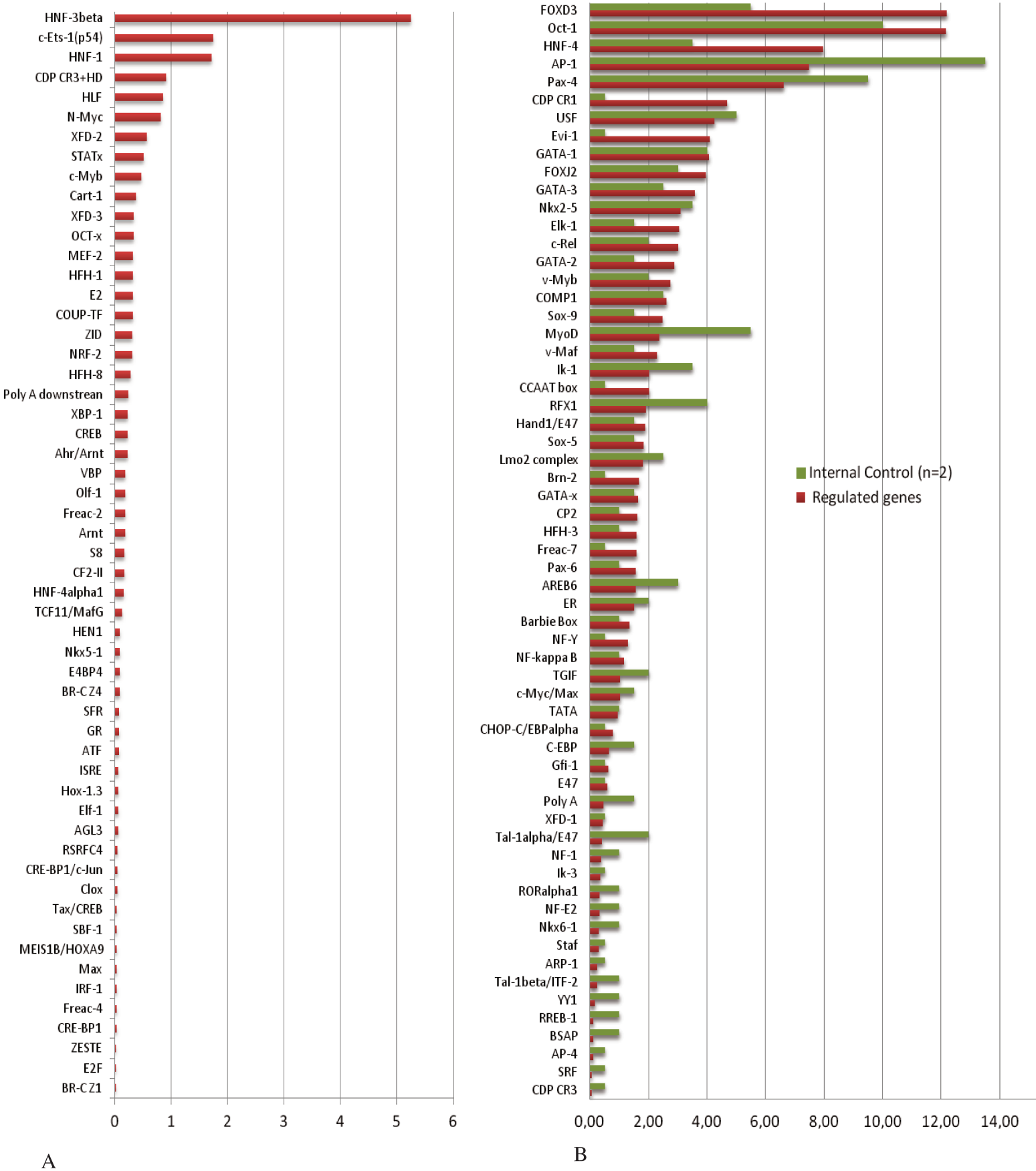

Figure 4. TF with possible binding sites found in the 80 most regulated genes exclusive and shared by the three experimental groups. (**A**) TF binding sites not found in the internal control genes. (**B**) TF binding sites also found in the proximal promoter of with internal control genes (right). Density values ≤ 0.01 were omitted.

Supplementar table 1: Transcriptors regulator (TR) found in the network of genes present in the enchement terms.

Cas	E2	Cas and Cas+E2	Shared by three groups
HOXA10	ARID5B	ANKRD6	AATF
HOXC5	ARL2BP	ARID5B	ARNTL
HOXC6	ARNT	ASCL2	BRCA1
HTT	BRCA2	ATF4	BRCA2
ID2	CBX3	ATF6	BTAF1
IKZF2	CEBPB	ATF1	CALR
IRF5	CEBPE	ATXN1	CBL
IRF7	E2F1	BRCA1	CCNT2
IRF8	ELK1	BTG2	CLIP2
IRF9	ELK3	CBFB	EAF2
IRX5	ELK4	CBL	ECSIT
JUN	ERF	CDKN2A	ELL3
KDM5B	ERG	CDKN2C	FOS
MAX	FOXO3	CDX2	GLI1
MECP2	GTF2H4	CEBPA	GTF2H3
MEF2C	HNF4A	CEBPB	HNF4A
Meg3	HOXB8	CEBPD	HOXA9
MXD1	HOXD3	CEBPE	HOXB5
MXD3	HSF2	CERS2	HTT
MXD4	HSF1	CERS5	IRF7
MXI1	HTATIP2	CERS6	JUN
MYBL2	MAX	CITED1	KLF5
MYC	MKL2	CREB1	LIMD1
MYCBP	MTPN	CREB3L1	LOC100046341
MYOCD	MYC	CREBBP	LSR
MYOD1	MYCBP	CREBL2	MAFK
NFATC2	MYCL1	CTBP2	MED12
NFE2L2	MYCN	CTNNB1	MEN1
NFKB1	NEO1	CUX1	MNX1
NM1	NFKBIZ	E2F1	MSRB2
OVOL1	NPM1	E2F2	MYBL1
POLD3	NUPR1	E2F4	NFATC1

Continuation

Cas	E2	Cas and Cas+E2	Shared by three groups
RELA	SERTAD1	EGR1	NFYC
RCF1	SIM2	EGR2	NOTCH1
RPL7/L1	SIRT1	ELF3	NPM1
Runx1	SIRT2	ELF4	POLR2J2/3
SALL1	SOX7	EPAS1	PSMC3
SATB1	STAT3	ETS1	PSMC5
SIN3B	USF1	ETS2	RAD54L2
SMAD3	USF2	EZH2	RB1
SMAD4	YY1	FLI1	RBAK
SMAD7	ZFP36	FOS	SBNO2
SMARCA2	ZNF143	FOSL1	SMAD9
SNW1		FOXA2	SQSTM1
SP4		FOXG1	SUPT4H1
SPI1		FOXK1	TAF3
SRF		FOXM1	Taf12
STAT1		FOXO3	TAF1L
STAT4		FOXO1	TAF7L
TAF15		FOXQ1	TAF9B
TAF1B		FUBP3	TCEA3
TBX2		GATA1	TCEB1
TCEB1		GATA2	TGIF1
TEAD4		GPBP1	TP53
TOB1		HCLS1	TP73
TP63		HDAC1	TRIM24
TP73		HIC1	TRIP13
TP53		HIF1A	TSC22D1
WT1		HMG20B	ZNF197
XBP1		HMGA1	
YY1		Hmgb1	
ZEB2		HMGB2	
ZNF148		HMGN5	
ZNF175		HNF1A	

Cas	E2	Cas and Cas+E2	Shared by three groups
PTGES2	PSMD10	E4F1	NFE2L2
RB1	PURA	EAF2	NFX1
RELA	SERTAD1	EGR1	NFYC
RCF1	SIM2	EGR2	NOTCH1
RPL7/L1	SIRT1	ELF3	NPM1
Runx1	SIRT2	ELF4	POLR2J2/POLR2J3
SALL1	SOX7	EPAS1	PSMC3
SATB1	STAT3	ETS1	PSMC5
SIN3B	USF1	ETS2	RAD54L2
SMAD3	USF2	EZH2	RB1
SMAD4	YY1	FLI1	RBAK
SMAD7	ZFP36	FOS	SBNO2
SMARCA2	ZNF143	FOSL1	SMAD9
SNW1		FOXA2	SQSTM1
SP4		FOXG1	SUPT4H1
SPI1		FOXK1	TAF3
SRF		FOXM1	Taf12
STAT1		FOXO3	TAF1L
STAT4		FOXO1	TAF7L
TAF15		FOXQ1	TAF9B
TAF1B		FUBP3	TCEA3
TBX2		GATA1	TCEB1
TCEB1		GATA2	TGIF1
TEAD4		GPBP1	TP53
TOB1		HCLS1	TP73
TP63		HDAC1	TRIM24
TP73		HIC1	TRIP13
TP53		HIF1A	TSC22D1
WT1		HMG20B	ZNF197
XBP1		HMGA1	
YY1		Hmgb1	
ZEB2		HMGB2	
ZNF148		HMGN5	
ZNF175		HNF1A	
ZNF217		HNF4A	
ZNF281		HOXA10	
ZCAN21		HOXC6	
		HTT	
		ID4	
		IRF2	
		IRF4	

Cas	Cas and Cas+E2
ZNF217	HNF4A
ZNF281	HOXA10
ZCAN21	HOXC6
	HTT
	ID4
	IRF2
	IRF4
	IRF7
	IRF8
	IRF1
	JUN
	IRF7
	IRF8
	IRF1

Continuation

Cas and Cas+E2	Cas and Cas+E2	Cas and Cas+E2
JUN	REL	XBP1
KCTD1	RELA	YWHAB
KDM5B	RUNX1	YY1
KEAP1	Runx1	ZBTB16
KLF2	RUNX2	ZBTB17
KLF6	RUNX3	ZFP36
KLF11	SATB1	ZFP57
LOC728622/SKP1	SIRT1	ZKSCAN1
MDM2	SMAD1	ZNF91
MECOM	SMAD3	ZNF140
MECP2	SMAD4	
MEN1	SMAD5	
MSRB2	SMAD7	
MXI1	SMARCA4	
Myb	SMARCB1	
MYBL2	SP1	
MYC	SP3	
MYCN	SP100	
NAB2	SPI1	
NCOA1	SQSTM1	
NCOA2	SREBF1	
NFATC2	SREBF2	
NFE2L2	SRF	
NFIA	STAT1	
NFKB1	STAT2	
NFKB2	STAT3	
NFYA	STAT4	
NFYB	STAT6	
NFYC	SUPT16H	
NKX3-1	TBR1	
NPM1	TBX2	
PAX3	TCEB1	
PDX1	TCF3	
PHB	TFDP2	
PITX2	TMF1	
PPARGC1A	TOB1	
PPARGC1B	TP53	
PREB	UBN1	
PTTG1	USF2	
PURA	VAV1	
RB1	WT1	

Appendix 1

Information about most regulated genes

Abcc3

Official Symbol/ Name: Abcc3/ATP-binding cassette, subfamily C (CFTR/MRP), member 3 [*Rattus norvegicus*]

Other Aliases: Mlp2, Mrp3

Other Designations: ATP-binding cassette sub-family C member 3; ATP-binding cassette, sub-family C (CFTR/MRP), member 3; MLP-2; MRP-like protein 2; canalicular multispecific organic anion transporter 2; multidrug resistance protein 3; multidrug resistance-associated protein 3; organic anion transporter

Chromosome/ Location: 10/10q26

Annotation: Chromosome 10, NC_005109.2 (82986552..83030799, complement)

Summary: human homolog transports conjugated metabolites from hepatocytes into the bloodstream; may play a role in steroid metabolism [RGD, Feb 2006]

Ankrd11

Official Symbol/Name: Ankrd11/ankyrin repeat domain 11 [*Rattus norvegicus*]

Other Designations: ankyrin repeat domain-containing protein 11

Chromosome/Location: 19/19q12

Annotation: Chromosome 19, NC_005118.2 (53208631..53242478, complement)

Summary (for Human): This locus encodes an ankryin repeat domain-containing protein. The encoded protein inhibits ligand-dependent activation of transcription. Mutations in this gene have been associated with KBG syndrome, which is characterized by macrodontia, distinctive craniofacial features, short stature, skeletal anomalies, global developmental delay, seizures and intellectual disability. Alternatively spliced transcript variants have been described. Related pseudogenes exist on chromosomes 2 and X. [provided by RefSeq, Jan 2012]

Aox3

Official Symbol/Name: Aox3/aldehyde oxidase 3 [*Rattus norvegicus*]

Other Aliases: Aoh1

Chromosome/Location: 9/9q31

Annotation: Chromosome 9, NC_005108.2 (56779880..56867683)

Aspn

Official Symbol/Name: Aspn/aspirin [*Rattus norvegicus*]

Other Aliases: RP11-77D6.3, OS3, PLAP-1, PLAP1, SLRR1C

Other Designations: asporin (LRR class 1); asporin proteoglycan; periodontal ligament associated protein 1; small leucine-rich protein 1C

Chromosome/Location: 9/9q22

Annotation: Chromosome 9, NC_000009.11 (95218487..95244844, complement)

Summary (for Human): This gene encodes a cartilage extracellular protein that is member of the small leucine-rich proteoglycan family. The encoded protein may regulate chondrogenesis by inhibiting transforming growth factor-beta 1-induced gene expression in cartilage. This protein also binds collagen and calcium and may induce collagen mineralization. Polymorphisms in the aspartic acid repeat region of this gene are associated with a susceptibility to osteoarthritis. Alternate splicing results in multiple transcript variants.[provided by RefSeq, Jul 2010]

Blnk

Interim Symbol/ Name: Blnk/ B-cell linker [*Rattus norvegicus*]

Other Designations: B-cell linker protein; cytoplasmic adapter protein

Chromosome/ Location: 1/1q54

Annotation: Chromosome 1, NC_005100.2 (246091335..246165075, complement)

Summary: (For Human) This gene encodes a cytoplasmic linker or adaptor protein that plays a critical role in B cell development. This protein bridges B cell receptor-associated kinase activation with downstream signaling pathways, thereby affecting various biological functions. The phosphorylation of five tyrosine residues is necessary for this protein to nucleate distinct signaling effectors following B cell receptor activation. Mutations in this gene cause hypoglobulinemia and absent B cells, a disease in which the pro- to pre-B-cell transition is developmentally blocked. Deficiency in this protein has also been shown in some cases of pre-B acute lymphoblastic leukemia. Alternatively spliced transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Jul 2008]

C1H6orf35

Official symbol: C1H6orf35 [*Rattus norvegicus*]

Other Aliases: rCG_40784, RGD1307262

Other Designations: uncharacterized protein LOC292228

Chromosome/Location: 1/1q11

Cdh12

Official Symbol/ Name: Cdh12 /cadherin 12 [*Rattus norvegicus*]

Other Aliases: RGD1566350

Chromosome/ Location: 2/2q21

Annotation: Chromosome 2, NC_005101.2 (72175690..72598944)

Summary (For Human): This gene encodes a type II classical cadherin from the cadherin superfamily of integral membrane proteins that mediate calcium-dependent cell-cell adhesion. Mature cadherin proteins are composed of a large N-terminal extracellular domain, a single membrane-spanning domain, and a small, highly conserved C-terminal cytoplasmic domain. Type II (atypical) cadherins are defined based on their lack of a HAV cell adhesion recognition sequence specific to type I cadherins. This particular cadherin appears to be expressed specifically in the brain and its temporal pattern of expression would be consistent with a role during a critical period of neuronal development, perhaps specifically during synaptogenesis. [provided by RefSeq, Jul 2008]

Cfh

Official Symbol/ Name: Cfh /complement factor H [*Rattus norvegicus*]

Other Aliases: Fh

Other Designations: complement component factor H; complement inhibitory factor H; platelet complement factor H

Chromosome/Location: 13/13q13

Annotation: Chromosome 13, NC_005112.2 (53252249..53355987, complement)

ID:155012

Summary: regulator of complement cascade activation [RGD, Feb 2006]

Chi3l1

Official Symbol/Name: Chi3l1/chitinase 3-like 1 (cartilage glycoprotein-39) [*Rattus norvegicus*]

Other Designations: CGP-39; GP-39; cartilage glycoprotein 39; chitinase 3-like 1 protein; chitinase-3-like protein 1

Chromosome/Location: 13/13q13

Annotation: Chromosome 13, NC_005112.2 (47139658..47147591)

Summary: human homolog plays a role in vascular smooth muscle cell migration and adhesion [RGD, Feb 2006]

Cntn6

Official Symbol/ Name: Cntn6 /contactin 6 [*Rattus norvegicus*]

Other Aliases: NB-3

Other Designations:contactin-6; neural adhesion molecule; neural recognition molecule NB-3

Chromosome/ Location: 4/4q41

Annotation: Chromosome 4, NC_005103.2 (140078007..140450348)

Summary: glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein that functions as a cell adhesion molecule; may play a role in axonogenesis [RGD, Feb 2006]

Cpa6

Official Symbol/ Name: Cpa6/ carboxypeptidase A6 [*Rattus norvegicus*]

Chromosome/ Location: 5/5q11

Annotation: Chromosome 5, NC_005104.2 (8287865..8403566)

Summary: (For Human). The protein encoded by this gene belongs to the family of carboxypeptidases, which catalyze the release of C-terminal amino acid, and have functions ranging from digestion of food to selective biosynthesis of neuroendocrine peptides. Polymorphic variants and a reciprocal translocation t(6;8)(q26;q13) involving this gene, have been associated with Duane retraction syndrome.[provided by RefSeq, Sep 2010]

Cyp2c13

Official Symbol: Cyp2c13 **(**Rattus norvegicus**)**

Official Full Name: cytochrome P450, family 2, subfamily c, polypeptide 13

Primary source: RGD:620363

Gene type:protein coding

RefSeq status: PROVISIONAL

Also known as Cyp2c38

Summary: polymorphic cytochrome P450 isozyme with male specific expression [RGD, Feb 2006]

Defb50

Official Symbol/Name: Defb50/defensin beta 50 [*Rattus norvegicus*]

Other Designations: BD-50; beta-defensin 50

Chromosome/Location: 16/16q12.5

Annotation: Chromosome 16, NC_005115.2 (74931529..74943827, complement)

Dmd

Official Symbol/Name: Dmd/dystrophin [*Rattus norvegicus*]

Other Aliases: DNADMD1, RATDMD

Other Designations: RNDNADMD1; apodystrophin-3; apodystrophin-I; dystrophin transcript variant Dp71e; dystrophin, muscular dystrophy

Chromosome/Location: X/Xq22

Annotation: Chromosome X, NC_005120.2 (71501362..71671414)

Summary: a vital component of a muscle sarcolemma membrane-spanning complex that connects cytoskeleton to basal lamina; plays a role in retinal neurotransmission; mutations cause Duchenne muscular dystrophy [RGD, Feb 2006]

Dync2h1

Official Symbol/Name: Dync2h1/dynein cytoplasmic 2 heavy chain 1 [*Rattus norvegicus*]

Other Aliases: Dnch2, Dnchc2

Other Designations: cytoplasmic dynein 2 heavy chain 1; cytoplasmic dynein heavy chain 2; dynein heavy chain isotype 1B; dynein, cytoplasmic, heavy chain 2; dynein, cytoplasmic, heavy polypeptide 2; dynein-like protein 4

Chromosome/Location: 8/8q11

Annotation: Chromosome 8, NC_005107.2 (3826843..4072644, complement)

Summary: human homolog is a dynein heavy chain involved in microtubule-dependent transport processes [RGD, Feb 2006]

Annotation information: Annotation category: partial on reference assembly

Furin

Official Symbol/Name: Furin/furin (paired basic amino acid cleaving enzyme) [*Rattus norvegicus*]

Other Aliases: Pace, Pcsk3

Other Designations: Paired basic amino acid cleaving enzyme (furin); Proprotein convertase subtilisin/kexin type 3 (paired basic amino acid cleaving enzyme, furin, membrane associated receptor protein); dibasic-processing enzyme; furin; paired basic amino acid residue cleaving enzyme; paired basic amino acid residue-cleaving enzyme; prohormone convertase 3; proprotein convertase subtilisin/kexin type3

Chromosome/Location: 1/1q31

Annotation: Chromosome 1, NC_005100.2 (136209969..136222057, complement)

Summary: may be one of several enzymes involved in the proteolytic processing of extracellular superoxide dismutase (EC-SOD) [RGD, Feb 2006]

Glrx1

Official Symbol/Name: Glrx1/glutaredoxin 1 [*Rattus norvegicus*]

Other Aliases: Glrx, Grx

Other Designations: TTase-1; glutaredoxin 1 (thioltransferase); glutaredoxin-1; thioltransferase-1

Chromosome/Location: 2/2q11

Annotation: Chromosome 2, NC_005101.2 (2888605..2899216)

Summary: catalyzes the deglutathionylation of protein-SS-glutathione mixed disulfides [RGD, Feb 2006]

Gnat2

Official Symbol/ Name:: Gnat2 / guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2 [*Rattus norvegicus*]

Other Designations: guanine nucleotide binding protein (G protein), alpha transducing 2; guanine nucleotide binding protein, alpha transducing 2; guanine nucleotide-binding protein G(t) subunit alpha-2

Chromosome/Location: 2/2q34

Annotation: Chromosome 2, NC_005101.2 (203652408..203655012)

Gpnmb

Official Symbol/Name: Gpnmb /glycoprotein (transmembrane) nmb [*Rattus norvegicus*]

Other Designations: osteoactivin; transmembrane glycoprotein NMB

Chromosome/ Location: 4/4q24

Annotation: Chromosome 4, NC_005103.2 (77161352..77182624)

Summary: may play a role in bone matrix production and mineralization; overexpressed in the osteopetrosis mutant op rat [RGD, Feb 2006]

Hamp

Official Symbol/Name: Hamp/hepcidin antimicrobial peptide [*Rattus norvegicus*]

Other Aliases: Hepc

Other Designations: hepcidin

Chromosome/Location: 1/1q21

Annotation: Chromosome 1, NC_005100.2 (85978120..85980059, complement)

Summary: may play a role in the regulation of iron metabolism [RGD, Feb 2006]

Annotation information: Annotation category: partial on reference assembly

Hectd2

Official Symbol/Name: Hectd2/HECT domain containing 2 [*Rattus norvegicus*]

Other Designations: probable E3 ubiquitin-protein ligase HECTD2

Chromosome/Location: 1/1q53

Annotation: Chromosome 1, NC_005100.2 (240791754..240835395)

Hspb6

Official Symbol/Name: Hspb6/heat shock protein, alpha-crystallin-related, B6 [*Rattus norvegicus*]

Other Aliases: Hsp20, p20

Other Designations: heat shock 20 kDa-like protein p20; heat shock 20-kDa protein; heat shock protein beta-6

Chromosome/Location: 1/1q21

Annotation: Chromosome 1, NC_005100.2 (85599075..85601214)

Summary: may play a role in muscle contraction [RGD, Feb 2006]

Ifit1

Official Symbol/Name: Ifit1/interferon-induced protein with tetratricopeptide repeats 1 [*Rattus norvegicus*]

Other Aliases: ISG56, Ifi56

Other Designations: GARG-16; IFI-56K; IFIT-1; glucocorticoid-attenuated response gene 16 protein; interferon-induced 56 kDa protein

Chromosome/Location: 19/19

Annotation: Chromosome 19, NC_000085.5 (34715379..34724499)

Summary: responses to dexamethasone and other inflammatory stimuli [RGD, Feb 2006]

Isyna1

Official Symbol/Name: Isyna1/inositol-3-phosphate synthase 1 [*Rattus norvegicus*]

Other Designations: IPS 1; MI-1-P synthase; MIP synthase; myo-inositol 1-phosphate synthase A1; myo-inositol-1-phosphate synthase

Chromosome/Location: 16/16p14

Annotation: Chromosome 16, NC_005115.2 (19345305..19348141, complement)

Summary (for Human): This gene encodes an inositol-3-phosphate synthase enzyme. The encoded protein plays a critical role in the myo-inositol biosynthesis pathway by catalyzing the rate-limiting conversion of glucose 6-phosphate to myoinositol 1-phosphate. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene, and a pseudogene of this gene is located on the short arm of chromosome 4. [provided by RefSeq, Nov 2011]

Kcnd2

Official Symbol/Name: Kcnd2/potassium voltage-gated channel, Shal-related subfamily, member 2 [*Rattus norvegicus*]

Other Aliases: KV4.2, RK5

Other Designations: potassium voltage-gated channel subfamily D member 2; voltage-gated potassium channel Kv4.2; voltage-gated potassium channel subunit Kv4.2; voltage-sensitive potassium channel

Chromosome/Location: 7/7q31

Annotation: Chromosome 7, NC_000007.13 (119913722..120390387)

Summary: a potassium channel responsible for transient outward K+ currents in cardiomyocytes [RGD, Feb 2006]

Kcnmb1

Official Symbol/Name: Kcnmb1/potassium large conductance calcium-activated channel, subfamily M, beta member 1 [*Rattus norvegicus*]

Other Designations: BK channel subunit beta-1; BKbeta; BKbeta1; calcium-activated potassium channel beta subunit; calcium-activated potassium channel subunit beta; calcium-activated potassium channel subunit beta-1; calcium-activated potassium channel, subfamily M subunit beta-1; charybdotoxin receptor subunit beta-1; k(VCA)beta-1; maxi K channel subunit beta-1; slo-beta; slo-beta-1; slowpoke-beta

Chromosome/Location: 10/10q12

Annotation: Chromosome 10, NC_005109.2 (18904902..18912604)

Summary: modulatory subunit of the MaxiK large conductance potassium channel; voltage and calcium-sensitive potassium channel [RGD, Feb 2006]

Krt15

Official Symbol/Name: Krt15/keratin 15 [*Rattus norvegicus*]

Other Aliases: Ka15

Other Designations: CK-15; K15; cytokeratin-15; keratin, type I cytoskeletal 15; keratin-15; type I keratin KA15

Chromosome/Location: 10/10q32.1

Annotation: Chromosome 10, NC_005109.2 (89071915..89075732, complement)

Summary: member of the keratin gene family; type I keratins are acidic proteins that heterodimerize with type II keratins in the formation of hair and nails [RGD, Feb 2006]

Lcn2

Official Symbol/Name: Lcn2/lipocalin 2 [*Rattus norvegicus*]

Other Aliases: Sip24

Other Designations: NGAL; alpha-2-microglobulin-related protein; alpha-2U globulin-related protein; lipocalin 2 (oncogene 24p3); lipocalin-2; neutrophil gelatinase-associated lipocalin; p25

Chromosome/Location: 3/3p11

Annotation: Chromosome 3, NC_005102.2 (11511402..11514747, complement)

Summary: mouse homolog plays a role in IL3 withdrawal-induced apoptosis [RGD, Feb 2006]

Ldoc1

Official Symbol/ Name: Ldoc1/ leucine zipper, down-regulated in cancer 1 [*Rattus norvegicus*]

Other Aliases: RGD1565644

Chromosome/ Location: X/Xq36

Annotation: Chromosome X, NC_005120.2 (147294439..147294882)

Summary: (For Human) The protein encoded by this gene contains a leucine zipper-like motif and a proline-rich region that shares marked similarity with an SH3-binding domain. The protein localizes to the nucleus and is down-regulated in some cancer cell lines. It is thought to regulate the transcriptional response mediated by the nuclear factor kappa B (NF-kappaB). The gene has been proposed as a tumor suppressor gene whose protein product may have an important role in the development and/or progression of some cancers. [provided by RefSeq, Jul 2008]

LOC259246

Official Symbol/Name: LOC259246/alpha-2u globulin PGCL1 [*Rattus norvegicus*]

Primary source RGD:708506

See related: Ensembl:ENSRNOG00000042641

Gene type: protein coding

RefSeq status: PROVISIONAL

Organism: Rattus norvegicus

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Sciurognathi; Muroidea; Muridae; Murinae; Rattus

Summary: member of a a highly homologous multigene family; expressed specifically in male liver and the coagulate glands [RGD, Feb 2006]

Annotation information: Annotation category: partial on reference assembly

LOC287167

Official Symbol/Name: LOC287167/globin, alpha [*Rattus norvegicus*]

Other Aliases: GloA

Other Designations: alpha-globin

Chromosome/Location: 10/10q12

Annotation: Chromosome 10, NC_005109.2 (15558815..15559543, complement)

LOC290071

Official Symbol/Name: LOC290071/similar to RIKEN cDNA A430107P09 [*Rattus norvegicus*]

Other Aliases: RGD1359684, Tcra, Tra29

Other Designations: T-cell receptor alpha chain

Chromosome/Location: 15/15p13

Annotation: Chromosome 15, NC_005114.2 (32234625..32273880)

LOC310926

Official Symbol/Name: LOC310926/hypothetical protein LOC310926 [*Rattus norvegicus*]

Primary source: RGD:1560890

Gene type: protein coding

RefSeq status: PREDICTED

Organism: Rattus norvegicus

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Sciurognathi; Muroidea; Muridae; Murinae; Rattus

Also known as: Aa1011; Aa1262; Ac1147; Ab2-057

Annotation information: Annotation category: partial on reference assembly

LOC689296

Description: similar to expressed sequence C79407 (LOC689296), mRNA [Source:RefSeq DNA;Acc:NM_001109531]

Location: Chromosome 6: 86,479,583-86,529,071 reverse strand.

Gene: This transcript is a product of gene ENSRNOG00000023093 - This gene has 1 transcript

LOC689295

Official Symbol/ Name: LOC689295/ hypothetical protein LOC689295 [*Rattus norvegicus*]

Chromosome/Location: 5/5q13

Annotation: Chromosome 5, NC_005104.2 (30767326..30773440, complement)

LOC691979

Official Symbol/Name: LOC691979/Similar to N-acetyltransferase ESCO2 (Establishment of cohesion 1 homolog 2) (ECO1 homolog 2) [*Rattus norvegicus*]

Other Aliases: Esco2

Other Designations: establishment of cohesion 1 homolog 2

Chromosome/Location: 15/15p12

Annotation: Chromosome 15, NC_005114.2 (45239871..45254898, complement)

Lrrc37a

Official Symbol/Name: Lrrc37a /leucine rich repeat containing 37A [*Rattus norvegicus*]

Other Aliases: Lrrc37a3, RGD1559753

Other Designations: RGD1559753; leucine rich repeat containing 37, member A3

Chromosome/ Location: 10/10q32.1

Annotation: Chromosome 10, NC_005109.2 (92637325..92756555, complement)

Lrrc34

Official Symbol/ Name: Lrrc34 /leucine rich repeat containing 34 [*Rattus norvegicus*]

Other Aliases: RGD1565052

Other Designations: leucine-rich repeat-containing protein 34

Chromosome/Location: 2/2q24

Annotation: Chromosome 2, NC_005101.2 (117173233..117193239)

Ly6c

Official Symbol/Name: Ly6c/Ly6-C antigen [*Rattus norvegicus*]

Chromosome/Location: 7/7q34

Annotation: Chromosome 7, NC_005106.2 (113179070..113182136, complement)

Summary: has similarity to mouse Ly6 antigens; may undergo posttranslational addition of a phosphatidylinositol-glycan membrane anchor [RGD, Feb 2006]

Macc1

Official Symbol/Name: Macc1/metastasis associated in colon cancer 1 [*Rattus norvegicus*]

Other Aliases: 7a5, RGD1309838

Other Designations: metastasis-associated in colon cancer protein 1; putative binding protein 7a5

Chromosome/Location: 6/6q33

Annotation: Chromosome 6, NC_005105.2 (146988261..147006788)

Summary (for Human): MACC1 is a key regulator of the hepatocyte growth factor (HGF; MIM 142409)-HGF receptor (HGFR, or MET; MIM 164860) pathway, which is involved in cellular growth, epithelial-mesenchymal transition, angiogenesis, cell motility, invasiveness, and metastasis. Expression of MACC1 in colon cancer (MIM 114500) specimens is an independent prognostic indicator for metastasis formation and metastasis-free survival (Stein et al., 2009 [PubMed 19098908]).[supplied by OMIM, Mar 2009]

Me1

Official Symbol/Name: Me1/malic enzyme 1, NADP(+)-dependent, cytosolic [*Rattus norvegicus*]

Other Aliases: MOD1

Other Designations: NADP-ME; NADP-dependent malic enzyme; cytosolic malic enzyme 1; malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+); malic enzyme 1, soluble

Chromosome/Location: 8/8q31

Annotation: Chromosome 8, NC_005107.2 (91839845..91955917, complement)

Summary: catalyzes the NADP+ dependent conversion of S-malate to pyruvate [RGD, Feb 2006]

MGC109340

Official Symbol/Name: MGC109340/similar to Microsomal signal peptidase 23 kDa subunit (SPase 22 kDa subunit) (SPC22/23) [*Rattus norvegicus*]

Primary source: RGD:1563536

Gene type: protein coding

RefSeq status: PROVISIONAL

Organism: Rattus norvegicus

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Sciurognathi; Muroidea; Muridae; Murinae; Rattus

Also known as: Spcs3

MMP12

Official Symbol: Mmp12 and **Name:** matrix metallopeptidase 12 [*Rattus norvegicus*]

Other Aliases: Mme

Other Designations: MMP-12; macrophage metalloelastase; matrix metalloproteinase 12; matrix metalloproteinase-12

Chromosome/ Location: 8/8q11

Annotation: Chromosome 8, NC_005107.2 (4249938..4259675)

Summary: a macrophage metalloelastase/matrix metalloproteinase; may have a role in glomerular injury in a crescentic glomerulonephritis [RGD, Feb 2006]

Mmp15

Official Symbol/ Name: Mmp15 / matrix metallopeptidase 15 [*Rattus norvegicus*]

Other Designations: matrix metalloproteinase 15

Chromosome/ Location: 19/ 19p13

Annotation: Chromosome 19, NC_005118.2 (10122553..10143853, complement)

Summary: (Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as

arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. However, the protein encoded by this gene is a member of the membrane-type MMP (MT-MMP) subfamily; each member of this subfamily contains a potential transmembrane domain suggesting that these proteins are expressed at the cell surface rather than secreted. [provided by RefSeq, Jul 2008]

Mrpl41

Official Symbol/Name: Mrpl41/mitochondrial ribosomal protein L41 [*Rattus norvegicus*]

Other Designations: 39S ribosomal protein L41, mitochondrial; L41mt; MRP-L41

Chromosome/Location: 3/3p13

Annotation: Chromosome 3, NC_005102.2 (5120299..5121217, complement)

Summary (for Human): Mammalian mitochondrial ribosomal proteins are encoded by nuclear genes and help in protein synthesis within the mitochondrion. Mitochondrial ribosomes (mitoribosomes) consist of a small 28S subunit and a large 39S subunit. They have an estimated 75% protein to rRNA composition compared to prokaryotic ribosomes, where this ratio is reversed. Another difference between mammalian mitoribosomes and prokaryotic ribosomes is that the latter contain a 5S rRNA. Among different species, the proteins comprising the mitoribosome differ greatly in sequence, and sometimes in biochemical properties, which prevents easy recognition by sequence homology. This gene encodes a 39S subunit protein that belongs to the YmL27 ribosomal protein family. [provided by RefSeq, Jul 2008]

N5

Official Symbol/Name: N5/DNA binding protein N5 [*Rattus norvegicus*]

Chromosome/Location: 4/4q22

Annotation: Chromosome 4, NC_005103.2 (57186821..57189020, complement)

Summary: potential DNA binding protein; may act as a transcription factor [RGD, Feb 2006]

Annotation information: Annotation category: partial on reference assembly

Nucleolin

Official Symbol/Name: Ncl/nucleolin [*Rattus norvegicus*]

Other Designations: nucleolin-like protein

Chromosome/Location: 9/9q35

Annotation: Chromosome 9, NC_005108.2 (85112752..85121276, complement)

Summary: nucleolar-specific protein; involved in cell growth [RGD, Feb 2006]

Neto2

Official Symbol/ Name: Neto2/ neuropilin (NRP) and tolloid (TLL)-like 2 [*Rattus norvegicus*]

Other Designations: brain-specific transmembrane protein containing 2 CUB and 1 LDL-receptor class A domains protein 2; neuropilin and tolloid like-2; neuropilin and tolloid-like protein 2

Chromosome/ Location: 19/19q11

Annotation: Chromosome 19, NC_005118.2 (22730832..22767097)

Summary: (For Human) This gene encodes a predicted transmembrane protein containing two extracellular CUB domains followed by a low-density lipoprotein class A (LDLa) domain. A similar gene in rats encodes a protein that modulates glutamate signaling in the brain by regulating kainate receptor function. Expression of this gene may be a biomarker for proliferating infantile hemangiomas. A pseudogene of this gene is located on the long arm of chromosome 8. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene. [provided by RefSeq, Jan 2011]

Obp3

Official Symbol/Name: Obp3/alpha-2u globulin PGCL4 [*Rattus norvegicus*]

Other Designations: alpha2 urinary globulin; odorant-binding protein 3

Chromosome/Location: 5/5q24

Annotation: Chromosome 5, NC_005104.2 (78088876..78092317, complement)

Summary: odorant binding protein and member of the alpha(2u)-globulin family [RGD, Feb 2006]

Annotation: Chromosome 1, NC_005100.2 (67115748..67116665, complement)

Summary not found

Ocm

Official Symbol/Name: Ocm/oncomodulin [*Rattus norvegicus*]

Other Aliases: OM, oncmodulin

Other Designations: parvalbumin beta

Chromosome/Location: 12/12p11

Annotation: Chromosome 12, NC_005111.2 (10916095..10925595)

Summary: calcium-binding protein; expression activated in a variety of tumors [RGD, Feb 2006]

Oas1k

Symbol: Oas1k

Full Name: 2 ' -5 ' oligoadenylate synthetase 1K

See related: Ensembl:ENSRNOG00000033220

Gene type: protein coding

RefSeq status: PROVISIONAL

Organism: *Rattus norvegicus*

Pi16

Official Symbol/Name: Pi16/peptidase inhibitor 16 [*Rattus norvegicus*]

Other Designations: protease inhibitor 16

Chromosome/Location: 20/20p12

Annotation: Chromosome 20, NC_005119.2 (7636282..7645221)

Pmp22

Official Symbol/Name: Pmp22/peripheral myelin protein 22 [*Rattus norvegicus*]

Other Aliases: Gas-3

Other Designations: PMP-22; SAG; SR13 myelin protein; schwann cell membrane glycoprotein

Chromosome/Location: 10/10q22

Annotation: Chromosome 10, NC_005109.2 (49305835..49335864)

Summary: mediates Schwann cell growth and peripheral myelin compaction; human homolog gene duplication causes Charcot-Marie-Tooth 1A (CMT1A) neuropathy [RGD, Feb 2006]

Annotation information: Annotation category: partial on reference assembly

Prol1

Official Symbol/Name: Prol1/proline rich, lacrimal 1 [*Rattus norvegicus*]

Other Aliases: Muc10, RSM-1

Other Designations: mucin 10, submandibular gland salivary mucin

Chromosome/Location: 14/14p21

Annotation: Chromosome 14, NC_005113.2 (21294053..21308816, complement)

Summary: a submandibular gland apomucin [RGD, Feb 2006]

RGD1562462

Similar to Ifi204 protein [*Rattus norvegicus*]

Official Symbol/Name: RGD1562462/similar to Ifi204 protein

Primary source: RGD:1562462

Gene type: unknown

RefSeq status: MODEL

Organism: Rattus norvegicus

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Sciurognathi; Muroidea; Muridae; Murinae; Rattus.

RGD1565682

Official Symbol: RGD1565682

Official Full Name: similar to lipase-like, ab-hydrolase domain containing 2.

Primary source: RGD:1565682

See related: Ensembl:ENSRNOG00000025444

Gene type:protein coding

RefSeq status:MODEL

Organism: *Rattus norvegicus*

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Sciurognathi; Muroidea; Muridae; Murinae; *Rattus.*

Location:1q52

Rnase2

Official Symbol/Name: Rnase2/ ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin) [*Rattus norvegicus*]

Other Aliases: EDN, RNS2

Other Designations: RNase 2; RNase UpI-2; eosinophil-derived neurotoxin; non-secretory ribonuclease; ribonuclease 2; ribonuclease US

Chromosome/Location: 14/14q24-q31

Annotation: Chromosome 14, NC_000014.8 (21423630..21424594)

Rnase1l1

Official Symbol/Name: Rnase1l1/ribonuclease, RNase A family, 1-like 1 (pancreatic) [*Rattus norvegicus*]

Primary source: RGD:1310106

Gene type: protein coding

RefSeq status: PROVISIONAL

Organism: Rattus norvegicus

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Sciurognathi; Muroidea; Muridae; Murinae; Rattus

Also known as: Rnase1; RNase 1; Rnase1d

Rpl21

Official Symbol/Name: Rpl21/ ribosomal protein L21 [*Rattus norvegicus*]

Other Aliases: RP11-428O18.5, L21

Other Designations: 60S ribosomal protein L21

Chromosome/Location: 13/13q12.2

Annotation: Chromosome 13, NC_000013.10 (27825692..27830705)

Summary: ribosomal subunit protein [RGD, Feb 2006]

Scnn1g

Official Symbol/Name: Scnn1g/sodium channel, nonvoltage-gated 1, gamma [*Rattus norvegicus*]

Other Aliases: ENaC

Other Designations: SCNEG; Sodium channel nonvoltage-gated 1 gamma (epithelial); Sodium channel, nonvoltage-gated 1, gamma (epithelial); amiloride sensitive sodium channel gamma1 subunit; amiloride-sensitive sodium channel subunit gamma; epithelial Na(+) channel subunit gamma; epithelial sodium channel gamma subunit; gamma-ENaC; gamma-NaCH; nonvoltage-gated sodium channel 1 subunit gamma; sodium channel, nonvoltage-gated, type I, gamma
Chromosome/Location: 1/1q36-q41
Annotation: Chromosome 1, NC_005100.2 (180555660..180589534)
Summary: acts as an epithelial sodium ion channel; regulates salt and fluid transport in the kidney [RGD, Feb 2006]
Annotation information: Annotation category: partial on reference assembly

Sdr16c5
Official Symbol/Name: Sdr16c5/short chain dehydrogenase/reductase family 16C, member 5 [*Rattus norvegicus*]
Other Aliases: RGD1565999, Rdhe2
Other Designations: epidermal retinal dehydrogenase 2; epidermal retinol dehydrogenase 2; retinal short chain dehydrogenase reductase 2
Chromosome/Location: 5/5q12
Annotation: Chromosome 5, NC_005104.2 (17339715..17361781, complement)

Sft2d1
Official Symbol/Name: Sft2d1/SFT2 domain containing 1 [*Rattus norvegicus*]
Other Designations: SFT2 domain-containing protein 1; vesicle transport protein SFT2A
Chromosome/Location: 1/1q11
Annotation: Chromosome 1, NC_005100.2 (47049663..47065559, complement)

Sh3rf2
Official Symbol/Name: Sh3rf2/SH3 domain containing ring finger 2 [*Rattus norvegicus*]
Other Aliases: Posh2, Ppp1r39

Other Designations: SH3 domain-containing RING finger protein 2; protein phosphatase 1 regulatory subunit 39; protein phosphatase 1, regulatory subunit 39; putative E3 ubiquitin-protein ligase SH3RF2

Chromosome/Location: 18/18p11

Annotation: Chromosome 18, NC_005117.2 (35242898..35344485)

Sparc

Official Symbol/Name: Sparc/secreted protein, acidic, cysteine-rich (osteonectin) [*Rattus norvegicus*]

Other Designations: BM-40; ON; **SPARC**; Secreted acidic cystein-rich glycoprotein (osteonectin); basement-membrane protein 40; osteonectin; secreted acidic cysteine rich glycoprotein; secreted protein acidic and rich in cysteine

Chromosome: 10/10q22

Annotation: Chromosome 10, NC_005109.2 (40809730..40831481, complement)

Summary: secreted structural protein; may be a component of collagen and the extracellular matrix [RGD, Feb 2006]

Slc25a24

Official Symbol/Name: Slc25a24/solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 24 [*Rattus norvegicus*]

Other Designations: calcium-binding mitochondrial carrier protein SCaMC-1

Chromosome/Location: 2/2q34

Annotation: Chromosome 2, NC_005101.2 (204721524..204760766)

Slc39a1

Official Symbol/Name: Slc39a1/solute carrier family 39 (zinc transporter), member 1 [*Rattus norvegicus*]

Other Designations: zinc transporter ZIP1

Chromosome/Location: 2/2q34

Annotation: Chromosome 2, NC_005101.2 (182490501..182496068)

Susd2

Official Symbol/Name: Susd2/sushi domain containing 2 [*Rattus norvegicus*]

Other Designations: sushi domain-containing protein 2

Chromosome/Location: 20/20p12

Annotation: Chromosome 20, NC_005119.2 (13435257..13442683, complement)

Ska3

Official Symbol/Name: Ska3 /spindle and kinetochore associated complex subunit 3 [*Rattus norvegicus*]

Other Aliases: RGD1307201

Other Designations: spindle and kinetochore-associated protein 3

Chromosome/ Location: 15/15p12

Annotation: Chromosome 15, NC_005114.2 (36848694..36867026, complement)

Summary: (For Human) This gene encodes a component of the spindle and kinetochore-associated protein complex that regulates microtubule attachment to the kinetochores during mitosis. The encoded protein localizes to the outer kinetechore and may be required for normal chromosome segregation and cell division. Alternative splicing results in multiple transcript variants. [provided by RefSeq, Oct 2009]

Slamf9

Official Symbol/Name: Slamf9/SLAM family member 9 [*Rattus norvegicus*]

Other Aliases: RP11-48O20.3, CD2F-10, CD2F10, CD84-H1, CD84H1, SF2001

Other Designations: CD2 family member 10; CD84 homolog 1; cluster of differentiation 2 antigen family member 10; cluster of differentiation 84 homolog 1; signaling lymphocytic activation molecule family member 2001; signaling lymphocytic activation molecule family member 9

Chromosome/Location: 1/1q23.2

Annotation: Chromosome 1, NC_000001.10 (159921282..159924044, complement)

Summary (for Human): This gene encodes a member of the signaling lymphocytic activation molecule family. The encoded protein is a cell surface molecule that consists of two extracellular immunoglobulin domains, a transmembrane domain and a short cytoplasmic tail that lacks the signal transduction motifs found in other family members. Alternative splicing results in multiple transcript variants.[provided by RefSeq, Apr 2009]

Slc17a9

Official Symbol/Name: Slc17a9/solute carrier family 17, member 9 [*Rattus norvegicus*]

Other Aliases: RGD1311940

Other Designations: solute carrier family 17 member 9

Chromosome/Location: 3/3q43

Annotation: Chromosome 3, NC_005102.2 (169820567..169837283)

Sms

Official Symbol/Name: Sms/spermine synthase [*Rattus norvegicus*]

Chromosome/Location: X/Xq21

Annotation: Chromosome X, NC_005120.2 (58816210..58870980)

Summary (for Human): The protein encoded by this gene belongs to the spermidine/spermine synthases family. This gene encodes an ubiquitous enzyme of polyamine metabolism. [provided by RefSeq, Jul 2008]

Tnnt2

Official Symbol/Name: Tnnt2/troponin T type 2 (cardiac) [*Rattus norvegicus*]

Other Aliases: CTTG, Ctt, RATCTTG, Tnnt3

Other Designations: Troponin T cardiac; cTnT; cardiac muscle troponin T; cardiac troponin T2; tnTc; troponin T, cardiac muscle; troponin T2, cardiac

Chromosome/Location: 13/13q13

Annotation: Chromosome 13, NC_005112.2 (48872972..48885815)

Summary: tropomyosin-binding subunit of troponin; confers calcium-sensitivy to actinomysin ATPase activity in striated muscle [RGD, Feb 2006]

Tns4

Official Symbol/Name: Tns4/tensin 4 [*Rattus norvegicus*]

Other Aliases: RGD1310402

Other Designations: tensin-4

Chromosome/Location: 10/10q31

Annotation: Chromosome 10, NC_005109.2 (87849160..87868697, complement)

Tmem47

Official Symbol/Name: Tmem47/transmembrane protein 47 [*Rattus norvegicus*]

Other Aliases: RGD1564799

Chromosome/Location: X/Xq22

Annotation: Chromosome X, NC_005120.2 (67522289..67549061)

Summary (for Human): This gene encodes a member of the PMP22/EMP/claudin protein family. The encoded protein is localized to the ER and the plasma membrane. In dogs, transcripts of this gene exist at high levels in the brain. [provided by RefSeq, Jul 2008]

Tomm7

Official Symbol/Name: Tomm7/translocase of outer mitochondrial membrane 7 homolog (yeast) [*Rattus norvegicus*]

Other Designations: translocase of outer mitochondrial membrane 7 homolog

Chromosome/Location: 4/4q11

Annotation: Chromosome 4, NC_005103.2 (6675775..6682616)

Summary (for Human): TOM7 is a small regulatory component of the translocase of the outer mitochondrial membrane (TOM), a general import pore complex that translocates preproteins into mitochondria.[supplied by OMIM, Apr 2004]

Twf1

Official Symbol/Name: Twf1/twinfilin, actin-binding protein, homolog 1 (Drosophila) [*Rattus norvegicus*]

Other Aliases: Ptk9

Other Designations: protein tyrosine kinase 9; twinfilin-1

Chromosome/Location: 7/7q35

Annotation: Chromosome 7, NC_005106.2 (133089255..133101150, complement)

Summary (for Human): This gene encodes twinfilin, an actin monomer-binding protein conserved from yeast to mammals. Studies of the mouse counterpart suggest that this protein may

be an actin monomer-binding protein, and its localization to cortical G-actin-rich structures may be regulated by the small GTPase RAC1. [provided by RefSeq, Jul 2008]

Ugt2b34

Official Symbol: Ugt2b34 and **Name:** UDP glucuronosyltransferase 2 family, polypeptide B34 [*Rattus norvegicus*]

Other Aliases: Ugt2b10

Other Designations: UDP glycosyltransferase 2 family, polypeptide B10; UDP-glucuronosyltransferase 2B10

Chromosome/ Location: 14/14p21

Annotation: Chromosome 14, NC_005113.2 (22725811..22745391)

V1rd15

Official Symbol/Name: V1rd15/vomeronasal 1 receptor, D15 [*Rattus norvegicus*]

Other Aliases: V1rd22, Vom1r33

Other Designations: vomernasal 1 receptor Vom1r33; vomeronasal V1r-type receptor V1rd22

Chromosome/Location: 1/1q12

Vom1r62

Official Symbol: Vom1r62

Official Full Name: vomeronasal 1 receptor 62

Primary sourceRGD:1359608

See related Ensembl:ENSRNOG00000032819

Gene type:protein coding

RefSeq status:PROVISIONAL

Organism:*Rattus norvegicus*

Lineage: Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Sciurognathi; Muroidea; Muridae; Murinae; Rattus.

Chromossome/ Location: 1/1q21

Wfdc3

Official Symbol/Name: Wfdc3/ WAP four-disulfide core domain 3 [*Rattus norvegicus*]

Chromosome/ Location: 3/3q42

Annotation: Chromosome 3, NC_005102.2 (155747773..155772497, complement)

Summary: (For Human) This gene encodes a member of the WAP-type four-disulfide core (WFDC) domain family. The WFDC domain, or WAP signature motif, contains eight cysteines forming four disulfide bonds at the core of the protein, and functions as a protease inhibitor. The encoded protein contains four WFDC domains. Most WFDC genes are localized to chromosome 20q12-q13 in two clusters: centromeric and telomeric. This gene belongs to the telomeric cluster. Alternatively spliced transcript variants have been observed but their full-length nature has not been determined. [provided by RefSeq, Jul 2008]

DISCUSSÃO

A próstata de ratos recebe constantemente a ação de diversos hormônios, dentre eles o andrógeno que é necessário para seu desenvolvimento, proliferação celular e sobrevivência. Quando há a interferência no processo de ação do andrógeno na próstata, sabe-se que há uma regressão do epitélio da próstata, remodelação do estroma e das células musculares lisas ajustando assim o tamanho do órgão à atividade reprodutiva como um todo (Vilamaior et al., 2000; Antonioli et al., 2005; 2007). Além disso, a privação do andrógeno desencadeia a morte das células epiteliais e um efeito antagonista sobre a proliferação celular (Isaacs 1984).

Dentre as diferentes maneiras para promover a privação androgênica existe o tratamento com estrógeno. Esse hormônio pode ser produzido através da conversão da testosterona (T) pela enzima aromatase (Simpson et al, 1999) e sua ação pode ser direta através dos seus receptores ERα e ERβ que estão distribuídos de forma diferenciada entre o epitélio e estroma (Weihua et al, 2002).

Está bem esclarecido que a castração através de cirurgia leva a próstata ventral de ratos a diminuir a quantidade de células epiteliais, coordenando a inibição da proliferação celular com a indução de morte celular apoptótica (Kryprianou e Isaacs, 1988; Powell et al 1996; Carvalho e Line, 1996; Carvalho et al, 1997; Powell et al, 1999; Vilamaior et al, 2000; Augusto et al, 2008). Já quando há a privação androgênica por estrógeno, há também a morte de células epiteliais na próstata porque tal hormônio interfere na produção de andrógeno pelos testículos inibindo a via hipotalâmica-hipofisária-gonodal (Huang et al, 2004).

Em nossos experimentos, analisamos as características morfológicas, proteínas da via AKT/PTEN e genes diferencialmente expressos perante a castração cirúrgica, a alta dose de 17β−estradiol e combinação de ambos os tratamentos na próstata ventral de ratos adultos tratados por três dias.

Os resultados nos mostraram que existem algumas características únicas de cada tratamento (castração, tratamento com estrógeno e a combinação de ambos os tratamentos) e outras que são compartilhadas.

O processo de descamação das células epiteliais foi observado nos grupos que envolvem castração (Cas e Cas+E2) e este é ausente nos outros grupos (CT e E2). Esse processo pode estar relacionado com a produção de enzimas proteolíticas tais como a Cathepsina-D, a qual se torna ativa a após a castração (Wilson et al, 1991). Tal achado morfológico pode ter relação com o

aumento de expressão gênica da MMP-15 encontrado nesses mesmos grupos, mesmo embora não tenhamos encontrado descamação epitelial no grupo E2.

Outro fenômeno que foi compartilhado por esses dois grupos de tratamentos foi a diminuição da produção da probasina, uma proteína básica produzida pela próstata como elemento de secreção do epitélio, inclusive das células luminais (Kasper et al, 2000). Observou-se sua alta redução pela análise de microarranjo de DNA nos tratamentos que envolvem a castração (fold-changes de -101 no grupo Cas e -39 no grupo Cas+E2). A diminuição da atividade secretora é um dos elementos relevantes na diminuição do peso relativo da próstata que também foi significativo para esses dois grupos, quando comparados com o controle.

Nos animais tratados com estrógeno (E2), observaram-se diversos aspectos sendo alguns presentes no grupo Cas+E2. Foi possível ver um aumento de camadas das células epiteliais, presença de divisão celular, dobramento do epitélio para a região interna do ácino o que justifica o aumento significativo na densidade de volume e volume absoluto do estroma determinado pela estereologia. Além disso, a metaplasia escamosa e a presença de agregados proteicos nas células epiteliais são características morfológicas compartilhadas com o grupo Cas+E2, assim como foi a identificação de células mitoticas. O fenômeno de estimulação da divisão celular e da metaplasia escamosa é conhecido como um resultado da exposição da glândula da próstata a altas doses de estrógeno ou de componentes estrogênicos (Risbridger et al, 2001) e parece atribuído ao receptor de andrógeno ERα (Chen et al, 2012). A presença de agregados proteicos nesses dois grupos é sugestiva de que proteínas que não foram enoveladas adequadamente causando assim uma resposta ao estresse de retículo e um indício para a participação desta via no desencadeamento para a apoptose (Xu et al, 2005; Woehlbier and Hetz, 2011).

Mesmo observando que as proteínas que participam na via AKT/PTEN não possuem em sua maioria uma diferença signifivativa nos grupos trabalhados, a proteína 4EBP em seu estado fosforilado teve uma redução significativa. A diminuição da fosforilação da proteína 4EBP ocorre através da proteína mTOR, a qual é sensível a níveis baixos de ATP na célula (Denis et al, 2001). Além disso, a hipofosforilção da 4EBP sequestra eIF4E e desfaz o complexo eIF4F (eIF4G-eIF4E-eIF4A) o qual é essencial para a tradução de RNAm que contêm regiões 5´-UTR longas e altamente estruturadas (Moerke et al, 2007), sendo que a maioria das proteínas de crescimento e proliferação possuem estruturas na região 5´-UTR (Lodish 1976; Pickering e Willis 2005). Então,

a diminuição da proteína 4EBP vista nos grupos que envolvem castração podem ser uma das causas para que tal fenômeno ocorra.

Os dados obtidos pela análise de microarranjo de DNA, nos mostrou, assim como na morfologia e bioquímica, que os tratamentos possuem respostas similares e exclusivas.

Os efeitos causados pela castração isoladamente ou combinada com o estrógeno envolve uma quantidade de genes maior do que aquele causado pelo estrógeno isoladamente. Isso porque, o número de genes que foram *up* ou *down* regulados é maior nesses dois grupos do que no estrógeno. Porém, o efeito combinado da castração com o estrógeno (Cas+E2) isoladamente, não é capaz de nos fornecer termos enriquecidos a partir dos genes regulados, somente com os mesmos genes compartilhados com os grupos E2 e Cas.

Outra diferença observada no perfil de expressão, é que no grupo E2 não há ontologias classificadas no processo de morte celular e apoptose. Portanto, os genes envolvidos no processo de morte celular pelo estrógeno são os mesmos que para os outros dois grupos de tratamentos.

Com respeito aos fatores de transcrição, o envolvimento e distribuição deles é bastante variada dentro dos sítios de ligação dos genes mais regulados dentro dos grupos exclusivos de cada tratamento como nos genes compartilhados pelos três tratamentos. Esse mesmo efeito é observado nos fatores de transcrição encontrados nas redes formadas com os genes enriquecidos.

A listagem final de fatores de transcrição (Evi-1, NF-Y, HNF-4, Elk-1, GATA-2, c-Rel, v-Myb and NFκB), mostra-nos uma distribuição diferenciada nas redes dos termos enriquecidos. Com respeito à distribuição nas regiões promotoras proximais dos genes mais regulados, existe uma distribuição variada estando em muitos casos concentrados em alguns genes de um grupo de tratamento específico e em outras vezes bem distribuídos.

De uma maneira geral, a distribuição dentro das redes dos termos enriquecidos, tem-se no grupo de genes compartilhados por Cas e Cas+E2 os fatores de transcrição: Evi-1; NF-Y; GATA-2 sendo da família de fatores de transcrição presentes na região responsiva ao andrógeno (ARE) *upstream* ao gene PSA (Schuur et al, 1996; Cleutjens et al, 1997; Pang et al, 1997; Zhang et al, 1997) e não expresso em células que não possuem AR, como a PC3 (Perez-Stable et al, 2000); v-Myb; NFκB que apesar de aparecer exclusivamente no grupo dos genes compartilhados pelos Cas e Cas+E2, apresenta uma alta densidade de ligação nos genes que são exclusivos do estrógeno e está envolvido em diversos processos de câncer de próstata (Suh et al, 2002; Zhang et al, 2009).

Elk-1 foi encontrado nas redes para os termos enriquecidos no grupo E2 e está relacionado com a via PI3K/AKT para a proliferação celular no carcinoma de pulmão (Zhang et al, 2006), o que corrobora com o fato do tratamento com estrógeno manter um estado proliferativo na próstata.

Portanto a presença de elementos que são diferenciados e/ou compartilhados pela castração através da alta dose de estrógeno ou por cirurgia é bastante notória e, portanto, os efeitos e mecanismos são pontos necessários de uma futura investigação. Dessa forma, a compreensão desses processos poderá auxiliar na escolha por uma terapia para o câncer de próstata com privação de andrógeno e anti-androgênica para que assim se possa prever melhor recorrência da doença e o desenvolvimento do câncer prostático independente de andrógeno.

CONCLUSÕES

1. A privação por andrógeno através da alta dose de estrógeno isolada ou combinada com a castração possui elementos de divisão celular associados;
2. Há o aspectos morfológicos exclusivos dos tratamentos como também elementos compartilhamento;
3. O tratamento da próstata ventral de ratos com a castração isolada ou combinada com o estrógeno gera uma resposta mais agressiva na produção de proteína de secreção e na hipofosforilação da proteína 4EBP;
4. Os fatores de transcrição encontrados tanto nos genes mais regulados de cada tratamento e compartilhados pelos três tratamentos como também na rede envolvendo genes dos termos enriquecidos, respondem de maneira diferenciada com relação aos tratamentos.

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Dissertação de Mestrado** intitulada **"REGRESSÃO PROSTÁTICA PÓS-CASTRAÇÃO: CARACTERIZAÇÃO DAS ALTERAÇÕES CAUSADAS PELA PRIVAÇÃO ANDROGÊNICA E ALTA DOSE DE 17β-ESTRADIOL"**:

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