UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

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O eixo heparam-sulfato/Heparanase1 na morfogênese epitelial de próstata

Heparan-sulfate/Heparanase1 axis on prostate epithelial morphogenesis

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Celular e Estrutural do Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

Doctorate thesis presented to the Cellular and Structural Biology Postgraduate Program in the Institute of Biology, the State University of Campinas, required for the completion of a Doctorate degree, in the area of Cellular Biology.

Orientador: Prof. Dr. Hernandes F. Carvalho

Este exemplar corresponde à versão final da tese defendida pelo aluno **Guilherme Oliveira Barbosa** e orientado pelo Dr. Hernandes F. Carvalho.

Assinatura do Orientador

Campinas, 2015

Agência(s) de fomento e nº(s) de processo(s): FAPESP, 2012/17657-0

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

Barbosa, Guilherme Oliveira, 1988-
B234e O eixo heparam-sulfato/Heparanase1 na morfogênese epitelial de próstata / Guilherme Oliveira Barbosa. – Campinas, SP : [s.n.], 2015.

Orientador: Hernandes Faustino de Carvalho.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Próstata. 2. Epitélio. 3. Morfogênese. 4. Heparanase. 5. Heparam sulfato. I. Carvalho, Hernandes Faustino de,1965-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Heparam-sulfate/Heparanase1 axis on prostate epithelial morphogenesis
Palavras-chave em inglês:
Prostate
Epithelium
Morphogenesis
Heparanase
Heparan sulfate
Área de concentração: Biologia Celular
Titulação: Doutor em Biologia Celular e Estrutural
Banca examinadora:
Hernandes Faustino de Carvalho [Orientador]
Enilza Maria Espreafico
Claudio Chrysostomo Werneck
Mário Henrique Bengtson
Maria Aparecida da Silva Pinhal
Data de defesa: 31-08-2015
Programa de Pós-Graduação: Biologia Celular e Estrutural

Campinas, 31 de Agosto de 2015

BANCA EXAMINADORA

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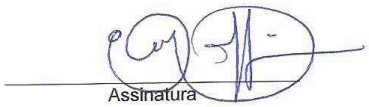
Profa. Dra. Enilza Maria Espreafico 
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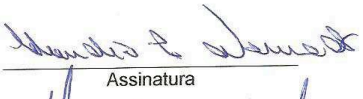
Prof. Dr. Claudio Chrysostomo Werneck 
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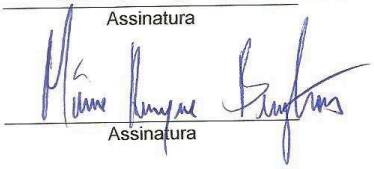
Prof. Dr. Mário Henrique Bengtson 
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Prefácio

Caro leitor, é com entusiasmo que exponho o meu trabalho de doutorado desenvolvido entre os anos de 2010 e 2015 no laboratório do professor Hernandes F. Carvalho. O trabalho intitulado "O eixo heparam-sulfato/Heparanase-1 na morfogênese epitelial de próstata" é composto por um resumo/abstract do material, seguido por uma introdução, que visa contextualizar aspectos básicos que justificam a relevância desse trabalho, bem como apontar o espaço no conhecimento de desenvolvimento de próstata de murinos que estamos preenchendo. Os achados desse estudo são expostos na forma de dois manuscritos e uma nota. Também escrevemos uma revisão (manuscrito 3) geral em que eu e Hernandes expomos algumas das ideias que temos sobre o nível de regulação que o heparam sulfato exerce na homeostase dos tecidos, com foque na próstata. Por fim, munidos desse conhecimento, me proponho a fazer uma discussão que visa especular futuros avanços para esse estudo, bem como extrapolar ideias/modelos de como o eixo Heparam sulfato/Heparanase-1 contribui nos diferentes aspectos da morfogênese prostática. Por fim em anexo apresento os materiais e métodos dos dois principais modelos de estudo utilizado nessa tese.

Esse trabalho só foi possível por que me apoiei ombros de gigantes para alcançar o que sou hoje. Poderia escrever um capitulo a parte sobre o professor Hernandes, mas vou tentar me resumir. Suas falas e ideias sempre aguçaram meus pensamentos. Acredito que meu envolvimento com o laboratório passaram pelos estágios necessários para garantir uma relação de confiança, amizade e profissionalismo, com a qual conduzimos nossas ações. Ainda aluno de graduação, ele orientou que eu estudasse dois artigos que imprimiram ideias e conhecimentos claros que conduziram minha pesquisa e forma de estudar desde então. *"MOTT, J. D.; WERB, Z. Regulation of matrix biology by matrix metalloproteinases. Current Opinion in Cell Biology"* me mostrou que entenderíamos a matriz extracelular do ponto de vista dinâmico, como um agente de regulação do comportamento das células. Esse artigo é assinado por Zena Werb com quem tive a possibilidade de discutir sobre matriz extracelular no Simpósio Internacional de Matriz Extracelular e na reunião da ASCB in 2011. *"MURAYAMA, A. et al. Epigenetic Control of rDNA Loci in Response to Intracellular Energy Status. Cell"* me mostrou que podemos atingir níveis de complexidade profundos onde há muito para se descobrir nos estudos de biologia celular. Professor Hernandes me colocou em contato com diferentes professores fora da UNICAMP os quais são sempre um prazer encontra-los nos eventos científicos, também foi através da ação dele que minha pesquisa pode

interagir com a professora Maria Aparecida da Silva Pinhal no estudo sobre heparam sulfato e Heparanase-1, com o professor Luis Lamberti Pinto da Silva sobre tráfico de vesículas e polarização celular, e com a professora Chao Yun Irene Yan sobre biologia do desenvolvimento. A esses professores sou grato pela receptividade em seus laboratórios e pela contribuição nos meus estudos e aprendizados. Em 2010, o Hernandes me envia para o *National Institute of Health*, ainda como aluno de graduação, para aprender com a equipe do Matthew Hoffman técnicas e aspectos da biologia de desenvolvimento de glândula salivar, nesse momento conheci Ivan Rebustini que pacientemente me guiou neste período e Sara Knox, que me instigou com suas perguntas interessantes sobre meus estudos. Também foi por intermédio do Hernandes que conversei com Richard O. Rynes na Reunião da SBBC em 2012 e recebi algumas dicas inspiradoras para o meu comportamento de busca enquanto cientista. Hernandes também estabeleceu uma parceria com o professor Carlos Lenz César ao criarem o Instituto Nacional de Fotônica Aplicada a Biologia Celular. Essa parceria me permitiu interagir com esse físico que desde então ilumina meus conhecimentos sobre fotônica e microscopia e também com a professora Mônica A. Cotta om quem elegantemente tenho discussões que aumentam minha curiosidades pelas ciências exatas para entender as células. Por fim, Hernandes me envia ao laboratório de Jeffrey D. Esko na Universidade da Califórnia San Diego nos EUA, onde me tornei um *"eskomo"* por seis meses aumentando meus conhecimentos sobre heparam sulfato, com foque no uso da técnica de edição do genoma, CRISPR/Cas9. Curiosamente antes de retornar ao Brasil, passei por Birmingham para visitar minha amada Suzana Ulian Benitez, e para completar minha viagem pelo mundo da glicobiologia tenho a possibilidade de conhecer a *School of Chemistry* na Universidade de Birmingham o local onde Sir. Norman Haworth realizou suas descobertas sobre as estruturas dos carboidratos, glicose e frutose, que o concedeu o premio Nobel em Química de 1937.

Tem como tudo isso ser perfeito? Ter colocado todo o meu fascínio por ciência sob orientação do professor Hernandes talvez tenha sido um dos meus grandes sucessos. Obrigado por me conduzir nos estudos, no trabalho e pessoalmente para quem sabe um dia poder atingir "o meu sonho Kepleriano". E sobre ser perfeito... Treinador Gaines, no final da ultima partida de futebol americano escolar, no filme *Friday Night Lights,* faz o discurso apropriado:

Well it's real simple: You got two more quarters and that's it.
Now most of you have been playin' this game for ten years. And you got two more quarters and after that most of you will never play this game again as long as you live.
Now, ya'll have known me for a while, and for a long time now you've been hearin' me talk about being perfect.
Well I want you to understand somethin'. To me, being perfect is not about that scoreboard out there. It's not about winning. It's about you and your relationship to yourself and your family and your friends.
Being perfect is about being able to look your friends in the eye and know that you didn't let them down, because you told them the truth. And that truth is that you did everything that you could. There wasn't one more thing that you could've done.
Can you live in that moment, as best you can, with clear eyes and love in your heart? With joy in your heart?
If you can do that gentlemen, then you're perfect.
I want you to take a moment. And I want you to look each other in the eyes. I want you to put each other in your hearts forever, because forever's about to happen here in just a few minutes…
Boys, my heart is full. My heart's full.

Agradecimentos

Às professoras Dra. Maria Aparecida da Silva Pinhal, Dra. Enilza Maria Espreafico e Dra. Helena Bonciani Nader, e aos professores Dr. Mário Henrique Bengtson, Dr. Claudio Chrysostomo Werneck, Dr. Ruy Gastaldoni Jaeger e Dr. Henrique Marques-Souza pela prestigiosa atenção, ao comporem a minha banca de defesa do Doutorado.

Ao programa de Pós-graduação em Biologia Celular e Estrutural, com particular atenção a Lílian Alves Senne pela sua atenção com os alunos e todos os coordenadores do programa.

À Universidade Estadual de Campinas, pela universalidade da minha formação.

À todos os professores do Instituto de Biologia da UNICAMP pela minha formação como biólogo. Em particular um agradecimento especial para Katlin Massirer, Daniel Martins De Souza, Fabio Papes e Marcelo Mori , além do Henrique e Mario acima citados, pelos estímulos acadêmicos e profissionais.

Aos amigos do IB, amigos da turma 07D pelo coleguismo fundamental na graduação e na vida.

Aos atuais e antigos membros do laboratório do professor Hernandes F. Carvalho, pelos esforços diários. Em especial Taize Machado Augosto, Alexandre Bruni Cardoso e Danilo Marchete Damas de Souza pelas suas inspiradoras experiências.

À FAPESP, pelo apoio financeiro para realização do meu doutorado (Processo n^{o} 2012/17657-0).

Na base do conhecimento, agradeço a todos os gigantes professores que me fizeram chegar até aqui, em especial professora Milena de S.B.O-SP, professora Jandira de Aracruz-ES e professor Teles de Americana-SP. Seus ensinamentos em ciências ecoam na minha cabeça até hoje.

Em se tratando de família, enalteço a coragem de minhas avos Dona Ana e Benedita e aos meus avos Zé Barbosa e Sebastião por isso meu grande agradecimento.

Fica o meu agradecimento a toda minha família, meus tios e primos, cuja presença na minha vida significa muito, em particular uma homenagem especial para minha tia Zezé, que nos deixou com uma enorme saudade no coração no meu “dedinho”.

Aos meus pais, eu atribuo todo o meu sucesso, são eles a minha fonte de inspiração, de acordar todos os dias e fazer cada vez melhor com tudo o que eles tem me ensinado a vida toda. Se hoje sou o que sou, foi por conta deles me que tornei. Obrigado Olivia Domingos de Oliveira Barbosa e José Valdemir Barbosa, não há orgulho maior no mundo do que poder dizer que sou filho de vocês dois. Essa inenarrável felicidade só é completa com a presença da minha irmã Mariana Oliveira Barbosa, meu orgulho.

Obrigado Kathryn Riley Ambler, “*my host mother, sister, friend”* de intercambio *in West Virginia*.

Dona Helena e Seu Nelson e Fernando, que me adotaram de braços abertos e coração maior ainda, ao namorar Suzana, a vocês o meu agradecimento. Seu Nelson nos deixou a mais de um ano cheios de saudade, e mesmo tendo convivido com ele por tão pouco tempo, uma coisa eu aprendi, a alegria, essa nunca pode faltar, nem nos momentos mais difíceis. Agradeço a cada segundo ter conhecido a Suzana Ulian Benitez, hoje minha namorada que eu amo, e com quem compartilho o amor, amor pela vida, amor pela ciência.

Para completar os demais amigos, amigos de longa data, de curta data, amigos inesquecíveis, amigos para todas as horas, meu muito obrigado por vocês existirem. Aproveito para deixar minha homenagem a Catharina Duarte Arnond, amiga que nos deixou neste período.

AOS MEUS PAIS OLIVIA E VALDEMIR

“I wanted to find the best conditions to fulfill my Keplerian dream. Dreams can be burdensome”

– Benoit B. Mandelbrot – The Fractalist

Resumo da tese

O desenvolvimento de próstata ventral (PV) de murinos inicia-se no dia embrionário 17,5. As células epiteliais do seio urogenital reduzem a expressão de Sulfatase-1 depois do primeiro pico de testosterona e mantêm a expressão de Heparam sulfato-6-O-Sulfotransferase-1. Essa combinação resulta no aumento do conteúdo de heparam sulfato (HS) altamente sulfatado, que é importante para sinalização de fatores parácrinos. A Heparanase-1 é uma endo-beta-D-glucuronidase responsável pelo *turnover* de HS nos tecidos dos vertebrados. Já que a próstata ventral remodela a matriz extracelular durante o desenvolvimento, elaboramos a hipótese que a Heparanase-1 e a sulfatação de HS possuem papel no desenvolvimento prostático pós-natal. Para testar nossa hipótese, usamos cultura de próstata ventral de rato e cultura de célula em 3D com matrigel de uma linhagem epitelial de próstata normal de humano, RWPE-1, que mimetiza a morfogênese acinar.

Detectamos a expressão de proteoglicanos de HS (HSPG), Syndecans e Glypicans, durante o desenvolvimento de PV. Tratamento com heparina ou silenciamento de *Hpse-1* atrasa o crescimento epitelial de PV e reduz a sinalização via ERK1/2. RWPE-1 diferencialmente expressam HSPG durante a morfogênese acinar. O tratamento com clorato, que reduz a sulfatação através da inibição da síntese de PAPS, nas células RWPE-1 em 3D elimina a formação de esferoides. O mesmo tratamento também inibe a canalização e morfogênese ramificada do epitélio de PV crescida *in vitro*, além de levar ao aumento de expressão de *Hpse-1* e *Mmp-2*. SDF-1, dentre diferentes fatores parácrinos parcialmente recuperou a formação de lúmen em ambiente com sulfatação reduzida. SDF-1 promove a formação e crescimento de esferoides em cultura 3D de RWPE-1, enquanto a inibição da sua sinalização, com AMD3100, inibe a organização das células. Por fim, o SDF-1 resgata a morfogênese acinar em ambiente pouco sulfatado. Concluindo, nossos resultados mostram que Heparanase-1 tem um papel importante no crescimento inicial do epitélio e a sulfatação é importante para canalização e morfogênese ramificada do epitélio durante o desenvolvimento pós-natal de PV. E SDF-1 atua na morfogênese epitelial mesmo em ambiente pouco sulfatado.

Thesis abstract

Murine ventral prostate (VP) development starts at embryonic day 17.5. Epithelial cells at the urogenital sinus down regulate the expression of Sulfatase-1 and keep the expression of Heparan Sulfate 6-O-sulfotransferase-1 in response to a testosterone peak. This combination increases highly sulfated heparan sulfate (HS) content, which is important for paracrine factors signaling. Heparanase-1 is an endo-beta-D-glucuronidase, responsible for the turnover of HS on vertebrate tissues. Because ventral prostate remodels extracellular matrix during development, we hypothesized that Heparanase-1 and sulfation of HS play a role on postnatal prostate development. To test our hypothesis we used rat ventral prostate organ culture and human normal prostate epithelial (RWPE-1) 3D cell culture on matrigel that mimics acinar morphogenesis. We detected the expression of HS proteoglycans (HSPG), Syndecan and Glipicans on VP during development. Either heparin or *Hpse-1* silencing delays epithelial growth, which also resulted in reduction of ERK1/2 signaling. RWPE-1 differentially expressed HSPG during acinar morphogenesis. Chlorate treatment, that reduces sulfation through PAPS synthesis inhibition, abolished RWPE-1 spheroids formation on 3D cell culture. Moreover it impairs epithelial canalization and branching morphogenesis of VP *in vitro*. Chlorate also increases the expression of *Hpse* and *Mmp-2*. SDF-1 partially recovers luminal formation in the low sulfated environment. SDF-1 also promotes spheroid formation and growth while its signaling inhibition with AMD3100 inhibits epithelial cell organization. SDF-1 rescued acinar morphogenesis under chlorate treatment. In conclusion, our results suggests that Heparanase-1 plays a role in early epithelial growth, that sulfation is of great importance for epithelial canalization and branching morphogenesis and that SDF-1 acts on epithelial morphogenesis even at low sulfated environment.

Sumário

Introdução

Os tecidos epiteliais ramificados exercem diversas atividades fisiológicas nos animais. Eles compartimentalizam regiões distintas nos órgãos e desempenham funções particulares: troca gasosa, produção de leite, produção de saliva, produção de enzimas digestivas, produção de componentes do liquido espermático, reabsorção de sais e agua, eliminação de metabólitos, circulação do sangue, formação do sistema imune inato, e outras funções que estão para ser descobertas ainda. Assim a formação da arquitetura de cada órgãos/tecidos correlaciona com o funcionamento, e ajuda entender o que acontece no desequilíbrio. Os tecidos epiteliais ramificados são originados a partir dos três folhetos embrionários, ectoderma, mesoderma e endoderma e apresentam características em comum: contato célula-célula definido por proteínas de junções, polarização apico-basal das células, e junções células-MEC com a membrana basal.

Neste trabalho buscamos entender alguns dos mecanismos por traz da morfogênese epitelial durante o desenvolvimento pós-natal de próstata de rato. No entanto há muito para entendermos sobre o estágio de desenvolvimento desse órgão, que mais é conhecido por acometer parte significativa da população masculina durante o final da fase adulta e no envelhecimento do que pela sua função fisiológica propriamente dita.

Origem embrionária do trato reprodutor masculino de mamíferos

As alterações decorrentes da diferenciação sexual primária, quando as gônadas são formadas, definem “duas biologias do desenvolvimento” a partir de um embrião indiferenciado na mesma espécie, a masculina e a feminina. Esse evento compromete o destino dicotômico do indivíduo desde a formação do corpo até o comportamento na sociedade.

Na gastrulação as células se diferenciam nas três linhagens de folhetos embrionário, ectoderma, mesoderma e endoderma. O mesoderma é responsável por gerar os órgãos contidos entre o ectoderma e o endoderma, bem como auxiliar na formação dos órgãos originados pelos demais folhetos embrionários. É no estágio nêurula que se inicia a diferenciação do mesoderma intermediário resultando na formação do sistema urogenital (rins), gônadas e os sistemas de ductos associados (GILBERT, 2010).

Os ductos mesonéfricos (Wolffianos) originam-se a partir do mesoderma intermediário e se ligam à região da cloaca. Os mesênquimas metanéfricos induzem uma ramificação em cada um dos ductos mesonéfricos, dando origem aos rins. O desenvolvimento desse outro órgão com epitélio ramificado é uma incrível historia a parte. Já os ductos paramesonéfrico (Müllerianos) surgem a partir da invaginacão do epitélio celômico, com localização adjacente aos ductos mesonéfricos, desembocando no tubérculo de Müller no seio urogenital. A cloaca é uma cavidade contínua do alantoide e conectada com o intestino primitivo posterior, ambos oriundos do endoderma. A projeção do septo urorectal em direção da membrana cloacal separa a cloaca do intestino primitivo posterior. Essa separação resulta na formação da bexiga e do seio urogenital a partir da cloaca (Fig. 1) (STAACK et al., 2003).

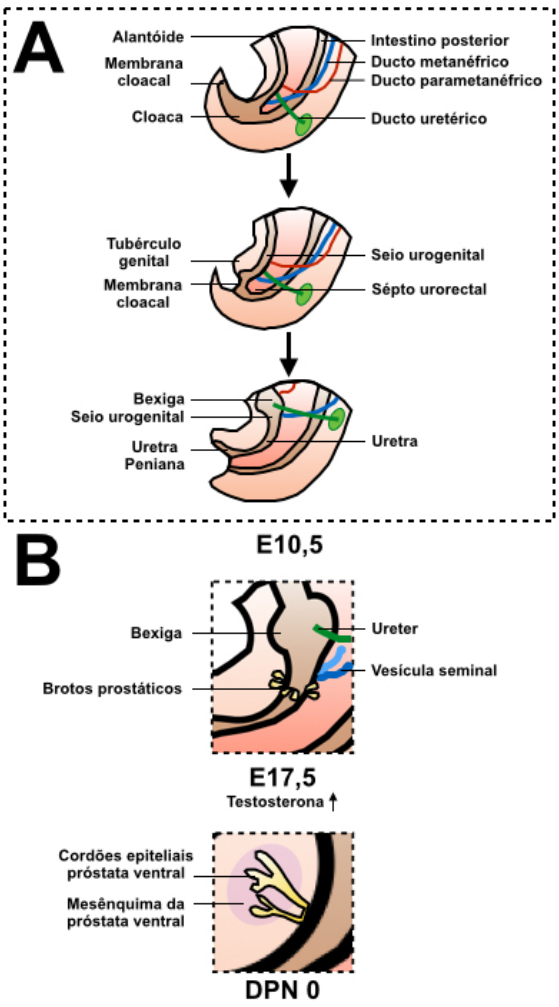

Figura 1. Desenvolvimento embrionário do seio urogenital de murinos. (A) A projeção do septo urorectal separa a cloaca do intestino posterior. (B) O pico de testosterona leva a indução prostática. Os brotos epiteliais invadem o mesênquima e formam os diferentes lobos prostáticos.

O evento inicial de distinção do sexo masculino é a diferenciação das células de Sertoli, a partir de células do sulco genital, em função da expressão do gene *Sry*, presente no cromossomo Y, e indução de expressão do gene *Sox9*. Assim essas células induzem a diferenciação de células mesonéfricas que migram para o sulco genital, em células endoteliais, peritubulares mioides e células de Leydig. Neste momento, células germinativas não diferenciadas também migram para a região do sulco genital onde organizam-se os precursores de cordões testiculares e as células germinativas se diferenciam em espermatogônias, também estimuladas pelas células de Sertoli.

As células de Sertoli já diferenciadas produzem hormônio anti-Mülleriano que atua na regressão do ducto paramesonéfrico. Já as células de Leydig passam a produzir testosterona responsável pela diferenciação dos ductos mesonéfricos em epidídimo, em vasos deferentes, e em vesícula seminal, também pelo desenvolvimento do tubérculo genital em pênis, e pela indução prostática a partir do seio urogenital, além das demais características masculinas (Fig. 2) (WILHELM; KOOPMAN, 2006).

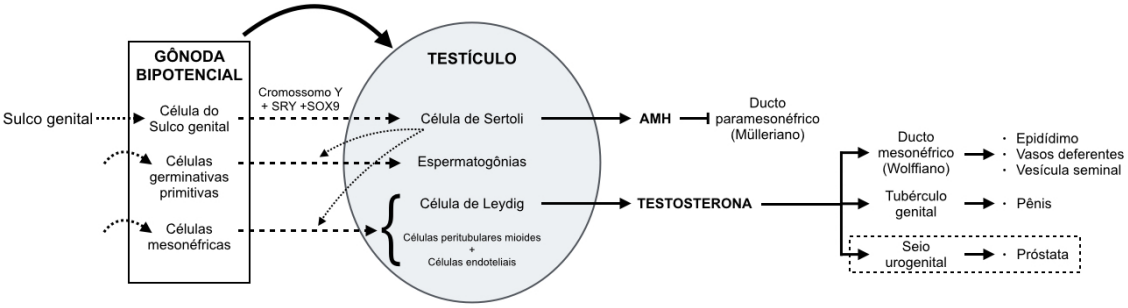

Figura 2. Esquema representativo dos eventos de diferenciação sexual primária, que resultam na diferenciação dos testículo, e consecutiva liberação dos hormônios anti-Mülleriano e testosterona, responsáveis pela diferenciação sexual secundaária, que envolve a masculinização do individuo e formação do trato reprodutor masculino.

Indução prostática em murinos

Com a diferenciação das células Leydig e o consequente aumento da produção de testosterona, inicia-se a indução prostática a partir do seio urogenital no dia embrionário 17,5 (E17,5). O mesênquima ao redor do seio urogenital é responsivo ao estimulo androgênico, e dessa forma medeia a indução prostática por meio de sinalização parácrina (CUNHA, 1972, 1973; CUNHA et al., 1983).

Os primeiros brotos epiteliais emergem na forma de cordões compactos de células epiteliais que se projetam na direção do mesênquima ao redor do seio urogenital. Cada um desses brotos alcançam mesênquimas especializados dando origem a diferentes lobos prostáticos: ventral, dorsolateral e anterior (TIMMS; MOHS; DIDIO, 1994).

Estima-se que haja uma ou mais moléculas, designada genericamente “andromedina” que seriam produzidas pelo mesênquima, mediante estimulação androgênica, capaz de induzir a formação do epitélio prostático a partir das células epiteliais do seio urogenital, mesmo na ausência de testosterona. Isso porque as células epiteliais do seio urogenital/próstata não expressam o receptor de andrógeno (AR) funcional na indução prostática (COOKE; YOUNG; CUNHA, 1991; HAYWARD et al., 1996). Uma questão curiosa e sem resposta ainda é qual seria o mecanismo

de regulação da expressão do receptor de andrógeno pelas células do mesênquima periuretral.

Desenvolvimento prostático pós-natal em murinos

O desenvolvimento da próstata dos murinos segue por aproximadamente 21 dias após o nascimento, estágio em que ocorrem as maiores transformações (Fig. 3A) (VILAMAIOR; TABOGA; CARVALHO, 2006): crescimento e projeção do epitélio nos diferentes estromas, inicialmente na forma de um cordão compacto de células; diferenciação das células epiteliais luminais, basais, neuroendócrinas e progenitoras (KARTHAUS et al., 2014; OUSSET et al., 2012); canalização mediada por secreção polarizada e morte celular e consequente alargamento do lumem (BRUNI-CARDOSO; CARVALHO, 2007; PEARSON et al., 2009); ramificação das estruturas epiteliais resultando na glândula epitelial do tipo túbulo-alveolar ramificada (SUGIMURA, 1986); e diferenciação das células mesênquimas em células musculares lisas circunjacentes ao epitélio, fibroblastos e das demais células constituintes desse tecido conjuntivo (THOMSON, 2008).

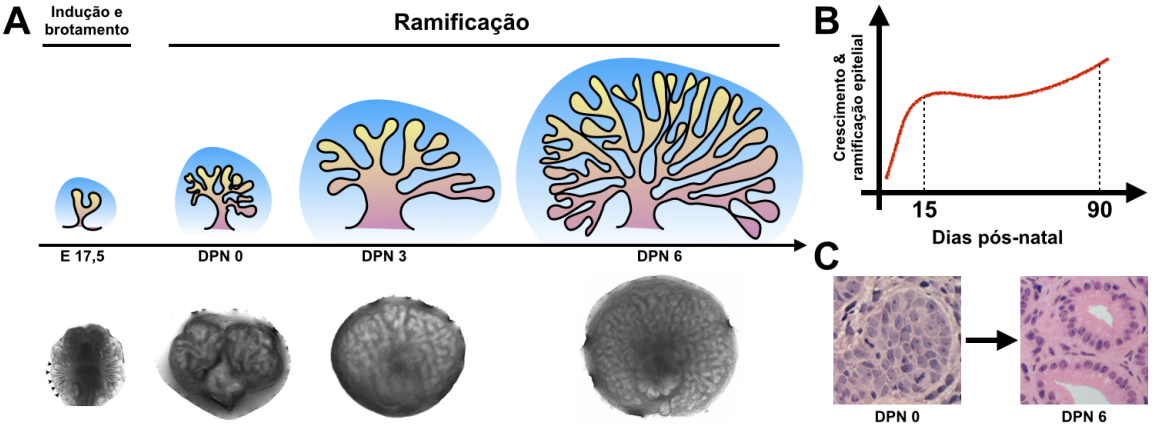

Figura 3. Desenvolvimento de próstata de murinos. (A) A cima esquema representativo da morfogênese da próstata de murinos com destaque para o lobo ventral, abaixo imagens tiradas em microscópio de campo claro representando os diferentes dias do desenvolvimento, que inicia no E 17,5 e segue por volta de 21 dias pós-natal (DPN). A maior parte do crescimento e ramificação do epitélio se da no período de 15 dias pós-natal, período em que as grande modificações no epitélio ocorrem (SUGIMURA, 1986). (C) Transformação do cordão epitelial compacto em ductos de epitélio simples colunar.

Por volta de 90 % dos ductos epiteliais nos três lobos prostáticos são formados em até quinze dias pós-nascimento (SUGIMURA, 1986) (Fig. 3B). A transição de cordão epitelial compacto para o epitélio simples colunar acontece na primeira semana pós-

natal. Essas talvez sejam duas das principais transformações do epitélio que irão resultar na glândula funcional no adulto (Fig. 3C).

Nosso grupo mostrou que as metaloproteinases de matriz-2 e 9 (MMP) exercem papel de remodelação de matriz extracelular durante o desenvolvimento pós-natal de próstata ventral de rato. Essas se localizam no estroma e nas extremidades distais dos epitélio, respectivamente (BRUNI-CARDOSO et al., 2008). Dessa forma, o bloqueio da expressão ou da atividade de MMP-2 compromete o crescimento epitelial; reduz a proliferação das células; altera a organização do epitélio, e resulta no acúmulo de fibras de colágeno no estroma (BRUNI-CARDOSO et al., 2010a, 2010b).

As andromedinas, sinalização parácrina e o SDF-1α

Descobrir qual mecanismo leva à indução prostática talvez seja um dos grandes mistérios na biologia do desenvolvimento deste órgão. Os candidatos mais explorados para cumprir esse papel foram o FGF10 e 7 (HUANG et al., 2005; PU et al., 2007; THOMSON; CUNHA, 1999; THOMSON, 2001). Essas moléculas são sintetizadas pelo estroma nas regiões próximas às zonas de crescimento epitelial durante a indução embrionária, assim como na fase de crescimento e ramificação pós-natal. O tecido epitelial dos três lobos prostático expressam receptores para esses ligantes (FGFR2iiib). No entanto somente os lobos anteriores e dorsolaterais são induzidos em camundongos com deleção condicionada no gene Fgfr2 ($Fgfr2^{flox/flox}$/Nkx3.1-Cre), o lobo ventral da próstata é ausente nesse animal. Os lobos que desenvolvem possuem todas as características de próstata, têm o crescimento e a secreção comprometido e são minimamente responsivos às alterações hormonais de testosterona, seja por castração ou aplicação no animal, mesmo expressando o receptor de andrógeno, o que demonstra que as sinalizações mediadas por FGF10 ou 7 e testosterona são importantes para o desenvolvimento da homeostase do órgão (LIN et al., 2007).

Os receptores de FGF dimerizam na membrana celular ao interagirem com seu ligante. Esse processo leva à auto-fosforilação de domínios intracelulares do receptor, que por sua vez desencadeia uma cascata de sinalização intracelular normalmente via proteínas quinases ativadas por mitógenos (MAPKs). No caso, tanto a inibição da fosforilação do receptor de FGF-10 e 7, quanto das MAPKs ERK1/2 inibe a indução prostática além do crescimento inicial do epitélio no órgão. No entanto os comprometimentos em crescimento e ramificação são menores se os

inibidores são aplicados 4 dias pós-natal (KUSLAK; MARKER, 2007). Tanto testosterona quanto FGF10 sozinho falham na indução prostática do seio urogenital de animais cujo gene Fgf10 é mutado. Somente com a combinação dos dois o evento de indução é possível (DONJACOUR; THOMSON; CUNHA, 2003).

Há uma série de outros fatores que devem ter papel na interação epitélio-mesênquima/estroma. Alguns desses fatores de sinalização parácrina são positivamente modulados nas células do estroma prostático, quando são co-cultivadas com células epiteliais prostáticas em matriz 3D, entre eles: SDF-1 α, NRG1, HGF, BMP5, PTN, TGFB2, FGF10, GMFG, PDGF e IL10. As células epiteliais por sua vez possuem receptores para alguns desses ligantes, SDF-1, FGF10 e TGF-β, sendo então responsivas ao estimulo do estroma (CHAMBERS et al., 2011).

SDF-1 sinaliza através de um receptor acoplado à proteína-G (GPCR) denominado CXCR4. Essa sinalização é muito associado à migração de células no estagio embrionário e células do sangue em diversas situações como inflamação (MILLER; BANISADR; BHATTACHARYYA, 2008). AMD3100 é um fármaco utilizada para auxiliar no tratamento de linfoma e mieloma agudo bloqueando a sinalização SDF-1 pelo receptor CXCR4. Essa droga foi utilizada para mostrar que SDF-1 tem papel no desenvolvimento ramificado de rins, afetando-o, e reduzindo a formação de glomérulos, que se dá através de transição mesênquima-epitélio (UELAND et al., 2009). Já no desenvolvimento de pâncreas, assim como em glândulas salivares, o receptor é expresso no epitélio enquanto o ligante no mesênquima e a sinalização leva ao desenvolvimento ramificado do epitélio assim como a formação de lúmen no pâncreas (HICK et al., 2009).

Significativa parte dos fatores de sinalização parácrina se liga à matriz extracelular quando liberados pelas células. Em particular ao heparam sulfato, que exerce outro nível de regulação no papel desses fatores. O ligante de HS deve ser capaz de ligar com heparina e com HS em condições fisiológicas de força iônica e pH. Na sua grande parte a interação entre proteína e HS é de natureza iônica. Os resíduos de lisina e arginina, positivamente carregados, interagem com os grupamentos carboxila e sulfeto presentes no HS. Ligação de hidrogênio e não-iônica também contribuem para essa interação (XU; ESKO, 2014).

Heparan sulfato e Heparanase-1

O heparam sulfato (HS) é constituinte importante das superfícies celulares e da matriz extracelular. É no Complexo de Golgi onde se inicia sua síntese, onde os açúcares aminados e ácidos glucurônicos são adicionados a um tetrasacarídeo O-ligado ao residuo de serina do core proteico pré-sintetizado. Transferases de N-acetilglucosamina e Ácido glucourônico, N-acetilglucosamina N-Deacetilases/N-Sulfotransferase (NDST), Uronil epimerase e O-Sulfotransferases operam sequencialmente na síntese de HS. As enzimas com atividades sulfotransferase usam 3'-fosfatoadenosina-5'-fosfosulfato (PAPS) como substrato doador de sulfato para as reações. Assim, ao se inibir a síntese de PAPS com a adição de clorato de sódio nas células, reduz-se o nível de sulfatação de HS, principalmente os níveis de 6-O e 2-O sulfatação provenientes da ação das enzimas Hs-2-O-sulfotrasnferases e Hs-6-O-sulfotransferases, respectivamente, quando comparados aos níveis de N-sulfatação realizadas pelas enzimas NDSTs. Essa diferença ocorre em função da diferença pela afinidade com o substrato das diferentes enzimas de sulfatação (SAFAIYAN et al., 1999).

Os tecidos e células diferencialmente expressam as isoformas das enzimas editoras de HS, resultando em cadeias com diferentes comprimentos e distribuições distintas de grupamentos sulfato nos glicosaminoglicano (GAG) (SUGAHARA; KITAGAWA, 2002).

Sulfatases (Sulf1 e Sulf2) removem os grupos O-sulfatados de HS expostos no espaço extracelular. Baixa sulfatação do HS inibe o crescimento tumoral (DAI et al., 2005); inibe a interação com receptores (ESWARAKUMAR; LAX; SCHLESSINGER, 2005; LOO et al., 2001); reduz o acúmulo de fatores de crescimento ligantes de heparina na MEC e superfície celular, influencia a transdução de sinal por diferentes receptores (DREYFUSS et al., 2009; PELLEGRINI, 2001); e também afeta a expressão e atividade da heparanase-1 (HPSE-1) (PURUSHOTHAMAN et al., 2008; REILAND et al., 2004; SANDERSON et al., 2005).

Proteoglicanos de HS (PGHS) restringem a difusão de fatores de crescimento, promovem sinalização localizada, estabelecem gradientes de concentração e evitam a degradação dos fatores (YAN; LIN, 2009). A presença de HS pelos tecidos depende da expressão de PGHS (LIN, 2004). PGHS são ancorados na membrana por GPI ou por um domínio transmembrana, como os Glipicans e os Sindecans, respectivamente (COUCHMAN, 2010).

A Heparanase-1 é uma beta-endo-D-glucuronidase que cliva cadeias de HS em dissacarídeos consecutivos ou espaçados, em sítios com específicos padrões de

sulfatação (PETERSON; LIU, 2012). As células expressam HPSE-1 durante a metástase, na neovascularização e em processos inflamatórios, já que HS é um componente importante na superfície celular e da MEC (IOZZO; ANTONIO, 2001; KJELLÉN; LINDAHL, 1991). Células tumorais que expressam heparanase-1 tem maior potencial invasivo (ILAN; ELKIN; VLODAVSKY, 2006).

A sequência codificadora do gene Heparanase-1 codifica uma proteína de 543 aminoácidos com massa molecular de ~65 KDa. Um fragmento de 8 KDa (correspondente à região N-terminal) e outro de 50 KDa (correspondente à região C-terminal) são formados no lisossomo, após processamento proteolítico, que resulta na enzima ativa heterodimérica (COHEN et al., 2005). Apenas um gene Hpse-1 codifica a enzima ativa (HULETT et al., 1999; KUSSIE et al., 1999; TOYOSHIMA; NAKAJIMA, 1999; VLODAVSKY et al., 1999) e sua sequência é conservada em humanos, ratos, camundongos e outras espécies (GOLDSHMIDT et al., 2001; HULETT et al., 1999).

Heparam sulfato e Heparanase-1 na próstata

A modulação da sulfatação de HS correlaciona com proliferação epitelial da próstata no estagio embrionário induzida por andrógeno, assim como com a atividade secretora do órgão em humanos (DE KLERK; HUMAN, 1985). Mais recentemente estudos em seio urogenital de camundongo parecem explicar este evento. A testosterona produzida pelo testiculo modula negativamente a expressão de Sulfatase-1 no seio urogenital de macho no dia embrionário 17,5. Isso resulta em aumento relativo do conteúdo de HS tri-sulfatado no epitélio prostático em brotamento, que deve regular a indução prostática de diferentes formas. A inibição de sulfatação com a adição de clorato em cultura de seio urogenital e/ou na indução da expressão de Sulfatase-1, seja induzida por BMP-4 7 ou por trasnfecção, resulta na inibição da indução prostática (BURESH et al., 2010). Além disso, o epitélio prostático em diferenciação expressa Hs-6-O-Sulfotrasnferase1, ao passo que a expressão de Sulfatase-1 fica restrita ao mesênquima, que delimita a região dos brotos epiteliais a serem enriquecidas em HS mais sulfatado (BURESH-STIEMKE et al., 2012).

Em ratos adultos a PV expressa mais Heparanase-1 no período pós-castração, quando acontece uma intensa remodelação de MEC (AUGUSTO; FELISBINO; CARVALHO, 2008). E a região promotora desse gene na próstata é sujeita a metilação em sítios CpG quando o animal é tratado com estrógeno no período pos-nascimento (AUGUSTO; ROSA-RIBEIRO; CARVALHO, 2011).

Justificativa

A castração química ou por remoção cirúrgica dos testículos, resulta em níveis androgênicos reduzidos e faz com que a próstata regrida. O que é utilizado como estratégias terapêuticas contra as lesões que acometem a próstata (HUGGINS; CLARK, 1940). Durante a regressão da próstata há uma intensa remodelação da matriz extracelular. Isso envolve a participação de Heparanase-1, que localizada no epitélio, na região basolateral das células epiteliais nos grupos controle e passa a se localizar no estroma com a redução do estímulo androgênico e o aumento da atividade da Heparanase-1 correlaciona com redução de HS na MEC (Augusto et al., 2008). O processo de remodelação de matriz extracelular é importante para morfogênese dos órgãos (BONNANS; CHOU; WERB, 2014). Heparam sulfato sintetizado na forma de proteoglicanos são um dos importante constituintes da superfície celular e da matriz extracelular (BISHOP; SCHUKSZ; ESKO, 2007). Além dos diversos papeis que exercem na homeostase dos tecidos, regulam a atividade de sinalização parácrina dos fatores de crescimento e de diferenciação que tem afinidade por HS (XU; ESKO, 2014). Essa interação permite ao HS estabelecer gradientes de fatores, estabilizar padrões de difusão, atuar como co-receptores e determinar a disponibilidade dos ligantes para sinalizarem e assim ditarem os padrões de diferenciação celular e morfogênese dos tecidos (HÄCKER; NYBAKKEN; PERRIMON, 2005). Na indução prostática onde o nível de Sulfatase-1 é reduzido mediante estímulo androgênico, que combinado com a expressão de Hs6st1, resulta no aumento de HS trisulfatado, importante na regulação de sinalização extracelular (BURESH et al., 2010; BURESH-STIEMKE et al., 2012). Assim a regulação do padrão de sulfatação de HS (PATEL et al., 2008) assim como o seu *turnover* (PATEL et al., 2007) são de extrema importância no desenvolvimento dos órgãos, incluindo a próstata. O que ainda estávamos por entender é qual o papel do heparam sulfato e da Heparanase-1 durante o desenvolvimento pós-natal da próstata de ratos, estagio onde as maiores transformações acontecem, resultando na formação da glândula epitelial ramificada do tipo túbulo-alveolar.

Desta forma buscamos com esse trabalho estabelecer o papel da homeostase do heparam sulfato durante o desenvolvimento prostático pós-natal em ratos. Procuramos determinar o padrão de expressão de Heparanase-1 bem como o seu papel na primeira semana do desenvolvimento e interferir na expressão dessa enzima, alterando o *turnover* de HS, e testando seus efeitos nas vias de sinalização mediadas por fosforilação de ERK1/2. Também buscamos entender como o nível de

sulfatação nas células impactaria a transformação de uma estrutura epitelial compacta cordonal até a formação da estrutural ramificada túbulo-alveolar. Neste ponto tentamos identificar o papel de SDF-1 no estágio de diferenciação e organização do epitélio, e como sua dinâmica é alterada de acordo com os níveis de sulfatação das células. Por fim, identificamos os proteoglicanos de heparam sulfato diferencialmente expressos em células epiteliais prostáticas humanas quando em contato com a membrana basal, e iniciamos a busca para entender qual seria o papel de Syndecan-1 no processo de organização epitelial.

Manuscrito 1: Heparanase activity is required for the early postnatal prostate development

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Abstract

Ventral prostate (VP) morphogenesis starts at the end of embryonic development and follows postnatally for three weeks. Cell differentiation, proliferation and death results in epithelial morphogenesis, which includes growth, branching and canalization. Extracellular matrix (ECM) remodeling is required for VP morphogenesis and depends on the crucial role played by matrix metalloproteinases (MMPs). Heparan sulfate (HS) is synthesized on proteoglycans protein cores and both affects cell membrane and matrix structure and paracrine signaling. Heparanase (HPSE) is the only enzyme at the vertebrate genome capable to cleave HS. HPSE releases HS bioactive fragment and mobilizes growth factors from the ECM reservoir. However little is known about HS turnover and HPSE function during VP morphogenesis. In this study, we sought to investigate the role of HPSE on postnatal VP development. First we detected the expression of HSPG. Then we analyzed the expression and distribution of HPSE at the mRNA and protein levels, uncovering that the enzyme is predominantly expressed by the VP epithelium. Heparin treatment was employed to interfere with HS homeostasis within tissue and resulted in delayed epithelial growth. To better understand the role of HS homeostasis in VP morphogenesis, we knocked down *Hpse* in *ex vivo* VP cultures. Hpse knocking down was validated by qRT-PCR and confirmed by changes in HS length after size exclusion chromatography. siRNA caused a delay of epithelial growth at the first postnatal week, which was similar to the treatment with heparin at low concentration. *Hpse* silencing resulted in up regulation of *Mmp9* and down regulation of *Mmp2* expression. It also down modulated ERK1/2 phosphorylation, which indicates that paracrine signaling is reduced, likely due to decrease in HS cleavage and growth factor bioavailability. In conclusion, HPSE plays a role in early epithelial growth during the first week of VP postnatal development.

Keywords

Prostate, heparanase, epithelial morphogenesis, development

Introduction

Ventral prostate (VP) development starts late in embryonic life, in response to a peak of testosterone production, and continues through three postnatal weeks in rodents (Cunha, 1973). Postnatal VP growth correlates with body growth indicating a somatotrophic regulation (Vilamaior et al., 2006). Epithelial cell mitosis occurs early in the first week (Sugimura et al., 1986). Apoptosis and polarized secretion contributes to canalization, which transforms the compact epithelial cord into hollow epithelial acini and ducts (Bruni-Cardoso and Carvalho, 2007; Pearson et al., 2009). Epithelial branching morphogenesis peaks at first postnatal week of development (Sugimura, 1986) when cell differentiation into the basal, luminal and neuroendocrine subtypes takes place (Karthaus et al., 2014; Ousset et al., 2012).

Growth and morphogenesis are accompanied by extracellular matrix (ECM) remodeling. Epithelial cells and the surrounding stromal cells organize the basal lamina and the ECM during prostate development. Cells deposit laminin and collagen fibers around the proximal growing epithelium, while cells at the distal tips seems to be constantly degrading the ECM, in order to invade the tissue. Matrix metalloproteinases (MMPs) play critical roles in this process. VP epithelium expresses *Mmp9* at the invading tips, and *Mmp2* at interface with the stroma (Bruni-Cardoso et al., 2008). Broad MMP inhibition and *Mmp2* silencing compromised epithelial growth and VP development resulting in the accumulation of hydroxyproline and collagen fibers (Bruni-Cardoso et al., 2010a, 2010b).

The glycosaminoglycan heparan sulfate (HS) is a linear polymer composed by the disaccharide N-acetylglucosamin-glucuronic/idouronic acid and is found both in the ECM and at the cell surface (Lindahl et al., 1998). Cells synthesize HS chains on proteoglycans and decorate it with sulfate at specific sites. The content and distribution of sulfate groups determine HS main properties, such as water recruitment, specific binding of many ligands, interaction with signaling receptors and substrate specificity (Bishop et al., 2007). Syndecans and glypicans are typical heparan sulfate proteoglycans (HSPG). HSPGs act as co-receptor, establish growth factor gradients, and signals through its cytosolic domain (Häcker et al., 2005).

HS turnover is finely coupled to the cellular processes. Heparanase is an endo-β-D-glucuronidase and is the only enzyme in the vertebrate genome known to cleave HS (Vlodavsky et al., 1999). It attaches the substrate through two specific domains (Levy-adam et al., 2005) and cleaves the chain at the beta linkage between glucuronic acid and 2-O-sulfated glucosamine (Peterson and Liu, 2012). *Hpse* knock out mice and mice overexpressing the human *HPSE* gene have normal development

and physiology. However, the *Hpse* KO mice have smaller and less branched mammary gland while the reverse is found in mice overexpressing the human *HPSE* (Zcharia et al., 2009, 2004, Gomes et al., 2015). Gomes et al. (2015) also showed that HPSE levels correlate with MMP14 expression to regulate mammary gland branching morphogenesis. HPSE releases FGF10 and HS bioactive fragments from perlecan in the basal membrane. The FGF10/HS complex signals for epithelial branching morphogenesis in the submandibular salivary gland (Patel et al., 2007). VP expresses more HPSE seven days after castration, and this increase correlates with the decreased HS content. The enzyme localization changes from basolateral at the epithelium to stromal, thus contributing to the ECM remodeling after castration (Augusto et al., 2008). HPSE promoter on VP is sensitive to newborn exposure to estrogen. The *Hpse* gene promoter becomes methylated at specific CpG, causing reduced expression in adults (Augusto et al., 2011).

In this work we aimed at determining the role of HPSE during VP postnatal development. Considering the importance of HS in the embryonic development of the prostate gland from the UGS epithelium (Buresh et al., 2010; Buresh-Stiemke et al., 2012), the androgen-regulation of HS content and HPSE location and activity in the adult organ (Augusto et al., 2008), not to mention the significance of HS for the proper timing and location of the particular signaling by different ligands taking part in the paracrine regulation of early postnatal prostate development, we hypothesized that interference with HS homeostasis (including the expression and activity of HPSE) would be mandatory for VP epithelial morphogenesis. In order to test this hypothesis we determine the expression of HSPG and HPSE *in vivo* and used VP cultures to interfere with HS homeostasis and to inhibit HPSE expression. Our finds demonstrated that Syndecans and Glypicans are differentially expressed in the organ within the first week of postnatal development, that heparin (employed to interfere with HS homeostasis, including HPSE blockade) retards epithelial growth, and that HPSE knocking down using siRNA delayed of epithelial growth and affected of *Mmp2* and *Mmp9* expression. We also showed that HPSE silencing resulted in reduced ERK1/2 activation. Thus HS and HPSE have a major role in VP early epithelial growth during the first week of postnatal development.

Materials and Methods

VP organ culture

Rat VP organ culture on floating porous membranes was performed as previously published (Bruni-Cardoso et al., 2010b). Heparin (Sigma-Aldrich, Saint Louis, MO, USA) was diluted in water and added to the culture medium at final concentration indicated in each experiment. siRNAs (Integrated DNA Technologies, Coralville, IA, USA) targeting *Hpse* mRNAs and Gfp (negative control) and Lipofectamin2000 (Invitrogen, Carlsbad, CA, USA) were prepared according to manufacture instructions. Briefly, Lipofectamine2000 was diluted in culture medium at 1:50 ratio, siRNA 10x in a different tube, after 50 µL of both tubes were mixture at 1:1 ratio and incubated in room temperature for 20 min. Finally, the solution was diluted 5 times and added to the well containing the membrane with the VP at final concentration indicated in each experiment. Medium was changed and micrographs taken on an inverted microscope AxioObserver every second day. Organs were kept in culture for 6 days. Animal-handling and experimental protocols were approved by the University's Committee for Ethics in Animal Experimentation (Protocol no. 2920-1).

Real-time PCR

Ilustra RNAspin Mini kit (GE Healthcare, Buckinghamshire, UK) was used to isolate RNA from a VP pool (at least three organs per group) according to the manufacture. Then RNA was transcribed into cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA) according to manufacture. Finally, approximately 20 ng of cDNA was used per well in a 20 µL reaction containing cDNA, distilled water, TaqMan 2x PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA) and Taqman inventoried assays. The assays used were Hpse (Rn 00575080_m1), b2-microglobulin (internal control for *in vitro* VP) (Rn00560865_m1) and HPRT (internal control for dissected VP) (Rn01527840_m1). The reaction was performed in a 7300 Real Time PCR System (Applied Biosystem, Foster City, CA, USA) and then ΔΔCt were calculated and the results expressed as fold-change relative to PND0 or Control group, depending on the experiment.

HS size exclusion chromatography

VP organ culture and *Hpse* silencing was performed as above. 40 µCi/mL of -(^{35}S)O_4^{-2} was added to the culture medium of every prostate for the last 18 hours of culture. Three VP per group were pooled and digested with 4 mg/mL of Maxatase-protease in 50 mM Tris-acetate buffer containing 1M NaCl at pH 8.0 for 24 hours at 60°C. Non-labeled HS was add to the mixture to help precipitate GAGs with two volumes of ethanol overnight. On the next day samples were centrifuged at 3000 RPM for 15 minutes and re-suspended in buffer containing 4 µU/µL chondroitinase ABC. Then each sample was analyzed by size exclusion chromatography in a Sepharose CL6B column of 25 mL total volume which the void volume and final volume were estimated with high molecular weight dextran blue and phenol red, respectively. The samples were collected in 500 µL fractions and the scintillations per minute were counted to estimate the HS content in the fraction.

Hpse mRNA *in situ hybridization*

VP were collected at postnatal days (PND) 0, 3 and 6 and frozen in Tissue-Tek OCT Compound (Sakura Finetek, USA). Eight µm sections were obtained and fixed with 4% PFA on glass slides. Fixed sections were washed in PBS and SSC 2x buffer (20x: 3M NaCl e 0.3M sodium citrate) and then digested with proteinase K (20 µg/mL) at room temperature before washed again in SSC 2x. After treatment with acetic anhydride solution (0.1M TEA, 5% acetic anhydride in DEPC water), and further wash in SSC 2x, sections were incubated in prehybridization buffer (50% formamide, 5X SSPE, 1X Denhardt's Solution (Life Technologies)) in a humidified chamber with 50% formamide in SSPE 5x (20x: 3M NaCl, 200 mM sodium fosfate and 20 mM EDTA, pH 7.4). The pre-hybridization buffer was replaced with new solution containing 100 ng of biotin-probe and 400 ng of tRNA overnight. On the next day sections were washed in SSC 4x and PBS and incubated in blocking buffer (100 mM Tris-HCl and 150 mM NaCl, pH 7.5) containing 0.3% Triton X-100 and 3% BSA. Then alkaline phosphatase-conjugated avidin (PIERCE, Rockford, IL, USA) (1:250) was added in blocking buffer, washed in phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl and 1g/L $MgCl_2$). Finally the enzyme activity was identified using NBT/BCIP at 1:1 ratio in phosphatase buffer and the reaction stopped with Stop Solution (10 mM Tris and 1 mM EDTA, pH 8). Sections were counterstained with methyl green and images were taken on Zeiss Axioskop microscope. Probe sequences were - Antisense1: Biotin – CACTCTTGACATTAACACCTTGGGACCTAC; Antisense2: Biotin – GTACAGAGTTAAATCTCCTTCCCGATACCTT; Antisense3: Biotin –

CTTCACTTATTTGCCTCTTGGTCATATTGG; Sense1: Biotin – GTAGGTCCCAAGGTGTTAATGTCAAGAGTG; Sense2: Biotin – AAGGTATCGGGAAGGAGATTTAACTCTGTAC; Sense3: Biotin – CCAATATGACCAAGAGGCAAATAAGTGAAG

Immunostaining

VP were collected at PND 0, 3 and 6 and frozen on TisseTek. Eight µm thick sections were obtained and fixed with 4% PFA on glass slides. Sections were washed in PBS, digested with Proteinase K (20 µg/mL) at room temperature and incubated in blocking buffer (3% BSA and 5% goat serum in PBS-T) and then incubated overnight with an antibody against HPSE1 (HPA-1, C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 in PBS-T containing 5% donkey serum). On the next day sections were washed in PBS and incubated with an AlexaFlour546-conjugated donkey-anti-goat Igs antibody (Life Technologies) (diluted 1:500) and stained with DAPI. Images where taken on Leica fluorescence microscope.

Western blotting

Five VP were pooled and used for protein extraction using RIPA buffer containing 1% protease inhibitor cocktail (Sigma Chemical Co.) and the protein quantity was estimated using the Bradford's method. Fifty µg protein per sample under reducing condition was loaded in 12% acrilamide gel. Electrophoresis was performed and proteins were wet-transferred to a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). Membrane was washed with TBS-T, blocked with 3% non-fat milk in TBS-T and incubated with an antibody against HPSE (HPA-1, C-20 SantaCruz Biotech.) (diluted 1:500) overnight in blocking solution. On the next day the membrane was incubated with a horseradish peroxidase-conjugated rabbit anti-goat IgG (diluted 1:5,000; Zymed-Invitrogen Laboratories, Carlsbad, CA, USA) and the peroxidase activity was identified with an enhanced chemiluminescent substrate (Santa Cruz Biotechnology). For ERK western blotting the same approach was employed with few modifications. Sodium orthovanadate and sodium fluoride were added to the protein extraction buffer to inhibit phosphatases and the antibodies used were p44/42 MAPK (Erk1/2) and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) diluted 1:2000 and 1:1000 respectively (Cell Signaling Technology, Danvers, MA, USA).

Data and image analysis

Data and statistical analysis were performed on Prim 6 for Mac OS X (1994 – 2014 GraphPad Software, Inc. La Jolla, CA, USA). The four-parameter logistic equation was used to fit curve on Growth index mean difference graph. The four parameters were estimated using Prims 6.

$$Growth\ index\ mean\ difference = Bottom + \frac{(Top - Bottom)}{1 + 10^{(TimeEC50 - Time) - HillSlope}}$$

Image processing and analysis were performed using ImageJ (Version 2.0.0-rc-31/1.49v, NIH, Bethesda, MD, USA).

Results

Rat VP expresses HSPGs during the early postnatal development

HS is part of different proteoglycans and plays pivotal roles during development. We investigated the expression of Syndecans and Glypicans in the rat VP at PND 0, 3 and 6 (Fig. 1) and found Syndecans 1, 2 and 4 and Glypicans 1-6 mRNAs in this period. Thus, the rat VP expresses different proteoglycans during the early postnatal development. Given the importance of HS and HSPG in signaling, they must contribute to the morphogenesis of the organ.

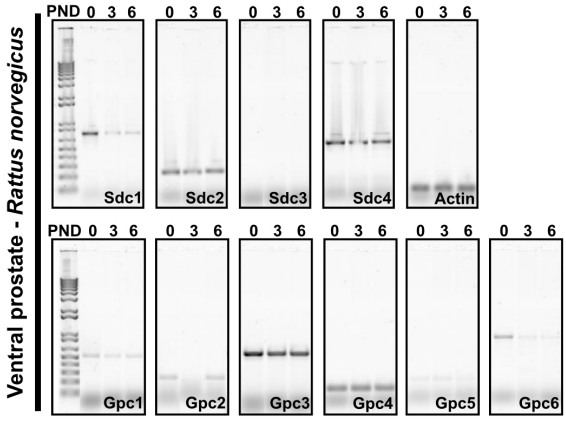


Fig 1. Syndecans 1-4 and Glypicans1-6 expression in the rat VP during the first week of postnatal development. Rat VPs at PND 0, 3 and 6 were collected and pooled (5 organs per time point) for RNA extraction. RT-PCR was performed to access the expression of the different Syndecans and Glypicans. Results reveal the expression of Syndecan 1, 2 and 4 and Glypican 1-6.

The VP epithelium expresses Hpse during the first week of postnatal development

HPSE cleaves HS and thereby affects the interaction of HSPG with HS-binding ligands and releases bioactive HS fragments. We reported before that HPSE expression shifted from the epithelium to the stroma in response to androgen deprivation (Augusto et al. 2008). Thus, we decided to investigate if the VP expresses HPSE during the first week of postnatal development. First we determined that the *Hpse* mRNA localizes predominantly on the epithelial structures (epithelial cords and acini) at PND 0, 3 and 6 (Fig. 2A). The relative content of *Hpse* mRNA decreases at PND 3 and 6, as compared to PND 0 (Fig. 2B). As expected from the mRNA localization, HPSE protein also localizes at the epithelial structures (Fig. 2C). HPSE was present in its pro-enzyme (65 kDa) and active forms (50 kDa) and its contents decreases from PND 0 to PND 6 (Fig. 2D). In line with our previous report

(Augusto et al. 2008), the present results suggest that epithelial expression of *Hpse* is under androgen regulation and that HPSE might play an important role on early epithelial morphogenesis and function based on its expression in the epithelial compartment.

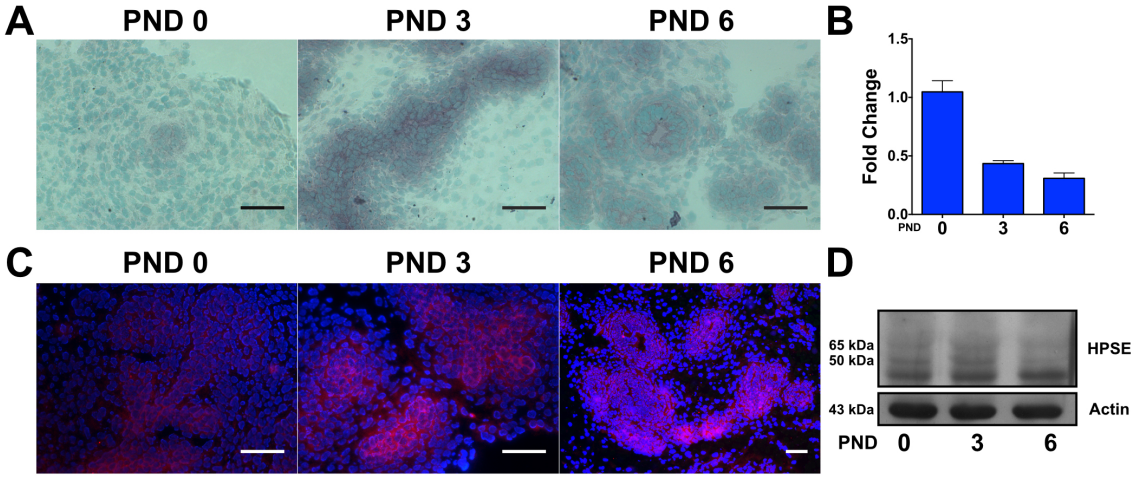


Fig 2. *Hpse* expression in the rat VP. (A) *In situ hybridization* showed the presence of *Hpse* mRNA in the VP epithelium during the first week of VP development; (B) mRNA relative content at PND 3 and 6 decreases as compared to PND 0; (C) immunostaining reveals the presence of HPSE in the epithelium. (D) Western blotting shows the the presence of HPSE precursor (65 kDa) and active forms (50 kDa) at PND0, 3 and 6. Scale bars in A and B = 50 µm. Bars on column graphs represent s.d. of the mean of technical triplicates of *pooled organs (n=5)*.

Heparin delays epithelial growth in VP in vitro

We treated VP *in vitro* with heparin to interfere on HS homeostasis during development (Fig. 3A). Heparin is a highly sulfated HS and can impact development by sequestering HS-binding factors from their local *reservoir*, unbalancing signaling pathways and inhibiting HPSE activity (Sasisekharan et al. 2002).

Both heparin concentrations (5 and 10 µg/mL) negatively affected epithelial growth at the forth day of culture (Fig. 3B). In addition, growth index mean difference of the heparin-treated groups as compared to control VP unveils distinct growth difference kinetics for each heparin concentration (Fig. 3C). The parameters suggest that the lower concentration had a major effect at day two of culture and the organ reaches a plateau of growth difference to control before day four, while the higher heparin concentration had a prolonged effect through the time line of the experiment with a shallower curve (HillSlope < 1), with continuous increase in growth difference to control (Fig. 3D). Altogether, these results imply that 5 µg/mL heparin interfere with an early mechanism of the VP epithelial growth *in vitro* and that the 10 µg/mL heparin concentration affects additional mechanisms.

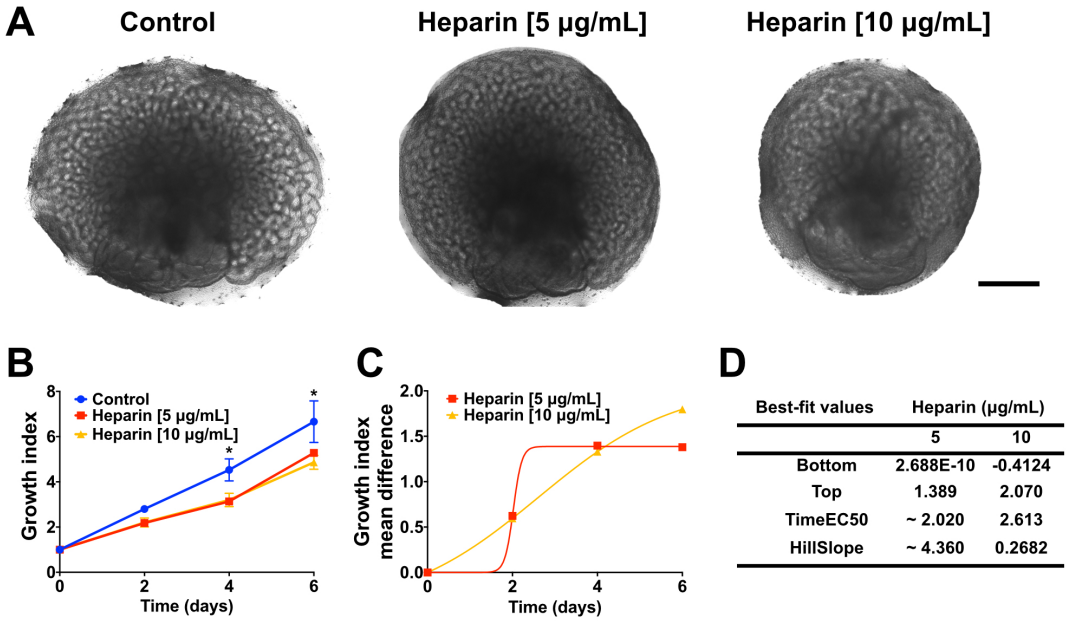


Fig 3. Effects of heparin treatment on VP growth *in vitro*. (A) VP images at PND6 reveals that heparin treatment *in vitro* delays epithelial growth. (B) Time-course growth index showed reduced growth at days four and six in response to heparin. (C) Growth index mean difference of heparin-treated VP compared to control fitted to a four-parameter logistic equation reveals distinct patterns in which either heparin concentration differentially affected epithelial growth. (D) Curve parameters table shows major difference on the Top value that indicates plateau level, a shift of around 0.6 day on TimeEC50 that indicates the period of inflexion of the curve and difference in HillSlope, which defines the steepness of the curve. These parameters indicate that 5 µg/mL heparin has its major effect around day 2 while 10 µg/mL heparin has negative effect on growth throughout the experiment time line and does not reach a plateau. Scale bar in A= 500 µm. Bars on line graphs (B) represent s.d. of mean (n=3 VP). Two-way ANOVA and Tukey's multiple comparisons test was performed and significance was assumed when $p<0.05$ (asterisks).

Heparanase knocking down compromises VP epithelial growth

We knocked-down *Hpse* expression in cultured VP, using siRNA to test if HPSE plays a role in epithelial growth. siRNA silencing at the two concentrations employed (50 and 100 nM), decreased *Hpse* mRNA relative content by ~50% and ~70% respectively, as compared to the control siGFP (Fig. 4A). ^{35}S labeled HS from VP was identified by size exclusion chromatography as two peaks with K_{av} values of 0.85 and 0.89 (arrow in Fig. 4B), indicating the presence of two major sizes of HS chains in the siGfp negative control (Fig. 4B). HPSE silencing resulted in the identification of a single peak with a clear left-shift corresponding to longer chain length, and disappearance of smaller chains in both knocked-down groups, further validating that *Hpse* silencing resulted in diminished HPSE activity (Fig. 4C). At both siRNA concentrations, *Hpse* silencing compromised epithelial growth as early as PND2 (Figs. 5A and B). The growth index mean difference of the *Hpse* silenced VP as compared to the siGfp negative control unveils similar kinetics of effect for both

siRNA concentrations (Fig. 5C). Curves fitting to the four-parameter logistic equation are steeper (HillSlope >1) with Top plateau reached around 1 before PND 2, albeit there is a significant shift in the time of inflection of the curve both indicates an early growth change (Fig. 5D) similar to what was observed treatment with 5 µg/mL heparin.

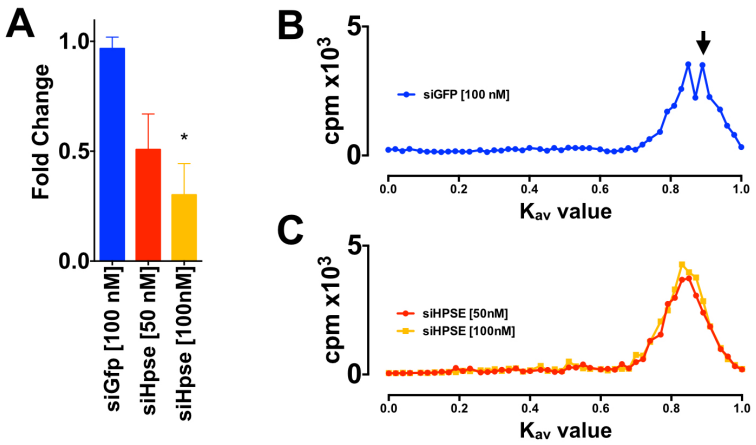


Fig 4. Validation of *Hpse* knock down and reduced enzyme activity after siRNA treatment (A) Real-time PCR demonstrates that the *Hpse* knockdown using either 50 or 100 nM siRNA for six days reduces the relative expression of *Hpse* in *ex vivo* cultured organs as compared to the negative control (siGFP). (B and C) ^{35}S labeled HS from the VP treated with control siRNA (siGFP) (B) or (C) *Hpse1* (50 and 100 nM siHpse). Gel exclusion chromatography on Sepharose CL6B after maxatase and chondroitinase ABC treatment. Two peaks were found in the control group and a single peak in the siHpse treated samples. The smaller chain length disappeared after *Hpse* knocking down and might represent the major product of HPSE activity. Hpse silencing produced a left shift, indicating longer HS chains. Bars on column graphs (A) represent s.d. of mean of biological duplicates of *pool* (3 VP each) per experimental condition. (A) One-way ANOVA and Tukey's multiple comparisons test were performed and significance was assumed when $p<0.05$ (asterisk).

Then we decided to investigate whether the expression of Mmp2 and 9 could be modulated with Hpse silencing. VP down regulates Mmp2 while an increases expression of Mmp9 (Fig. 6A) when siRNA silences Hpse expression. So Mmp2 mRNA content positively correlates with Hpse while Mmp9 negatively correlates (Fig. 6B). These results demonstrate that HPSE plays an important role in epithelial growth during the early stage of epithelial morphogenesis at the first week of postnatal VP development and that *Hpse* knocking down changes the expression patterns of *Mmp2* and *Mmp9*, and that the effect of the lower heparin concentration is predominantly resulted from its effect on HPSE inhibition.

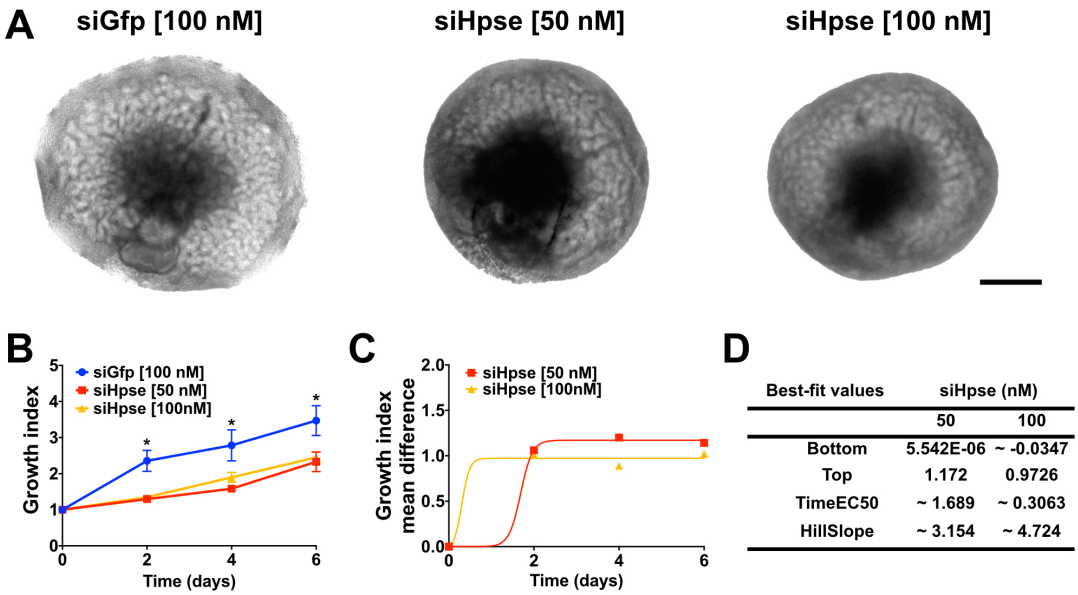
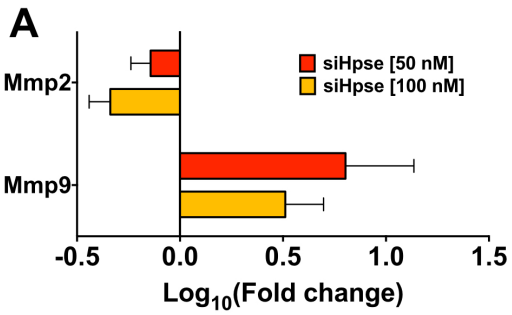


Fig 5. *Hpse* knock down affects VP growth *in vitro*. (A) *Hpse* knockdown using siRNA at the indicated concentrations retards epithelial growth. (B) Time-course growth index showed difference in epithelial growth from day two to day six. (C) Growth index mean-difference of siHpse knocked-down VP compared to siGfp negative control fitted to a four-parameter logistic equation reveals similar patterns of growth effect on the two concentrations of siRNA. (D) Curve parameters table shows major difference on TimeEC50, with a shift of around 1 day that indicates the period of inflexion of the curve and a in HillSlope, which defines the steepness of the curve. The parameters indicate that 5 µg/mL heparin has its major effect at some point around day 2 while 10 µg/mL heparin has a negative effect on growth throughout the whole period of time and does not reach a plateau. Scale bar: (A) 500 µm. Bars on line graphs represent s.d. of (B) mean of 6 VPs per group. Two-way ANOVA and Tukeys multiple comparisons test were performed and significance was assumed when $p<0.05$ (asterisks).



B

Pearson Correlation	r value	*p* value
Hpse - Mmp2	0.902	0.014
Hpse - Mmp9	-0.592	0.216
Mmp2 - Mmp9	-0.418	0.410

Fig 6. Differential expression of *Mmp2* and *Mmp9*. (A) Real-time PCR demonstrates that *Hpse* knockdown down regulates the relative expression of *Mmp2* and increases *Mmp9 ex vivo* as compared to the negative control (siGFP). (B) Table of Pearson correlation of gene expression reveals positive correlation between *Hpse* and *Mmp2 mRNA contents*, while there is a negative correlation between *Hpse* and *Mmp9*. No statistical significance was found after One-way ANOVA test (A).

ERK1/2 signaling is reduced after Hpse knocking down

There are many HS-binding ligands participating on paracrine interactions between the stroma and epithelium during VP development. One such paracrine factor is FGF10. Stromal cells express FGF10 that signals to epithelial cells through its receptor, FGFR2iiib (Huang et al. 2005). FGF10 (and other HS-binding ligands) signaling in epithelial cells funnels down into ERK1/2 phosphorylation.

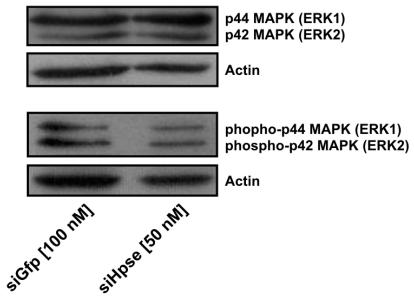


Fig 7. ERK-1/2 phosphorilation is reduced under *Hpse* silencing. Western blotting for total ERK1/2 and phospho-ERK1/2 revealed reduced signaling in the siHpse group.

Discussion

HS turnover plays pivotal role at early stages of epithelial growth during postnatal VP development. We show that in this period, the VP prostate expresses HSPGs Syndecans 1, 2 and 4 and Glypicans 1-6, *Hpse*, *Mmp2* and *Mmp9*. HSPGs are major components of cell membrane, basal lamina and ECM acting as structural components and regulators of extracellular signaling (Iozzo, 2005). The urogenital sinus (UGS) epithelium accumulates tri-sulfated HS due to the expression of HS-sulfotransferases and down regulation of Sulfatase1 upon androgen stimulation (Buresh et al., 2010; Buresh-Stiemke et al., 2012). Thus, HS expression and editing seem to be conditional for murine prostate development. Disrupted extracellular signaling resulting from the lack of a proper set of HS species abolishes VP induction and further morphogenesis. HPSE and the gelatinases (MMP2 and MMP9) are important for the homeostasis of HS and HSPG, respectively, and their expression suggest that HS/HSPG homeostasis play important roles on prostate development, particularly by affecting HS-binding ligands signaling.

The VP epithelium expresses HPSE, a modulator of HS content and integrity at the plasma membrane and ECM. The peak of expression happens at early stages of VP epithelial growth and branching morphogenesis similar to what happens on submandibular salivary gland (SMG) during embryonic development (Patel et al., 2007) where HPSE cleaves HS from perlecan in the basal membrane and releases HS bioactive fragments and FGF10 that favors epithelial growth and branching. We previously reported that the adult rat VP expresses HPSE, which localizes at the basal lateral portion of the epithelium. Its localization switches to the stroma and correlates to the decreased HS content within the tissue after castration (Augusto et al., 2008). Moreover Hpse gene promoter is prone to methylation due to estrogen exposure after birth, resulting in reduced gene expression in VP at adult life (Augusto et al., 2011). HPSE is present in non-tumoral human prostate epithelium, and its expression increases in prostate benign lesions and cancer, however, *Hpse* expression apparently decreases at the advanced stages of cancer progression (Stadlmann, 2003).

There is much information on the role of HPSE in cancer progression and invasion and angiogenesis (Ilan et al., 2006). However, little is known about the expression of Hpse during the development. A possible explanation is the absence of appealing phenotypes in the *Hpse* knockout mouse (Zcharia et al., 2009, 2004). This indicates the need for specific conditional KO or different approaches to better understand the role of this enzyme during development. More recently, a study described *Hpse*

expression in mammary gland and reported decreased growth and branching in the *Hpse* KO mouse and increased branching after *Hpse* overexpression (Gomes et al., 2015)

Heparin compromised VP epithelial growth *in vitro by* interfering with HS homeostasis. Heparin is a highly sulfated HS with potential roles in coagulation, immune modulation, P-selectin-mediated cell adhesion block, dislocation of signaling factors and inhibition of HPSE activity (Sasisekharan et al., 2002). It inhibits epithelial growth in rat lung morphogenesis *in vitro* (Shiratori et al., 1996) and in submandibular salivary gland without interfering in branching morphogenesis (Patel et al., 2008). Even though heparin affects multiple events, the *in vitro* system might reduce the possible targets. In the present experimental conditions heparin could affect morphogenesis mainly via unbalancing extracellular signaling of HS binding factor and inhibiting HPSE activity.

Hpse silencing with siRNA, validated by qRT-PCR and HS size exclusion chromatography, delayed epithelial growth at early stages of postnatal VP development and promoted changes in *Mmp2* and *Mmp9* expression. Similarly, inhibition of MMPs activity and *in vitro* silencing of *Mmp2* reduced VP epithelial growth, branching and cell proliferation with concomitant accumulation hydroxyprolin and collagen fibers in the ECM (Bruni-Cardoso et al., 2010a; Bruni-Cardoso et al., 2010b). Although it is not clear whether the difference in epithelial growth stagnates or still increases through the whole week *in vitro*, these results together indicate the existence of a time window at early stage of epithelial morphogenesis that demands on ECM turnover – in particular HS – and its disruption results in epithelial growth delay.

In this study we showed that HPSE silencing correlated with reduced ERK1/2 phosphorylation, suggesting a down modulation of extracellular paracrine signaling from the stroma that signals via ERK in the epithelium. Stromal cells differentially expresses chemokines, HGF, FGFs and TGFβs when co-cultured with epithelial cells in 3D (Chambers et al., 2011a). On the other hand epithelial cells become responsive to these signals under these conditions (Chambers et al., 2011b).

These paracrine factors are of great importance for prostate development, given the fact that only the UGS mesenchyme cells are responsive to androgen stimulation in early development (Cunha, 1973, 1972). Thus, it is believed that paracrine factors, such as FGF10, function as andromedins, i.e. signaling molecules regulated by testosterone on mesenchymal cells, that signal to the UGS epithelium to induce prostate formation. FGF10 signals duct elongation, epithelial branching and cell differentiation via the FGFR2iiib receptor (Huang et al., 2005), albeit FGF10 alone is

not sufficient to recover *in vitro* prostate bud formation in FGF10 KO UGS, and a combination with testosterone is necessary, indicating the need for additional stromal factors (Donjacour et al., 2003).

MAPK pathway is determinant for prostate induction and early epithelial growth, conveying signaling from FGFRiiib upon FGF10 stimulation. However it does not affect epithelial growth and branching late at the first week of postnatal development (Kuslak and Marker, 2007). Although not conclusive, this correlates with heparin and *Hpse* silencing major growth effect during early postnatal development.

In conclusion, the VP expresses HSPGs and the proper HS synthesis, editing and processing define the characteristic and dynamics of HS homeostasis during development. Disruption of HPSE function, using pharmacological inhibition with heparin or knocking down gene expression with siRNA, compromises epithelial growth *in vitro* during the first postnatal week and down regulation of ERK1/2 signaling pathway indicating the paracrine regulation of epithelial growth and morphogenesis is compromised. Finally, we suggest that impaired signaling due to reduced HPSE activity, affects the expression of the gelatinases *Mmp2* and *Mmp9.*

Acknowledgments

This work was funded by FAPESP (Grant no. 2009／16150-6) and CNPq. G.O.B was supported by a fellowship from FAPESP (no. 2012／17657-0).

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Manuscrito 2: SDF1 role on prostate epithelial morphogenesis unveiled important roles of heparan sulfate/heparan sulfate proteoglycans

Keywords: Heparan sulfate, sulfation, sodum chlorate, prostate, epithelial morphogenesis, branching, canalization, SDF1

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Abstract

Androgen induces murine prostate development at embryonic day 17.5. It down regulates Sulfatase1 on epithelium, which expresses Heparan Sulfate-6-O-Sulfotransferase1, resulting in tri-sulfated heparan sulfate (HS). Thus heparan sulfate (HS) editing seems important for paracrine signaling during rat ventral (VP) induction from the urogenital sinus epithelium. However the role of HS on postnatal epithelial morphogenesis was unknown. We have hypothesized that prostate epithelium morphogenesis needs proper HS sulfation and tested whether sodium chlorate would interfere with organogenesis in both RWPE-1 in 3D cell culture and in the *ex vivo* VP organ culture. Sodium chlorate inhibits PAPS synthesis, which is a sulfate donor for sulfotransferase activity. Sulfation reduction abolished acinar morphogenesis in 3D epithelial cell culture, inhibited epithelial canalization and branching and up regulated *Hpse1* and *Mmp2* expression in the VP *in vitro*. SDF1 induced epithelial canalization on distal tips in the low sulfated environment unveiling its role on epithelial morphogenesis. Furthermore, SDF-1 enhances acinar morphogenesis and recovers it under low sulfated environment. All together, the results show a significant role for sulfation on postnatal prostate epithelium morphogenesis and reveal a role for SDF1 in epithelial canalization.

Introduction

Prostate is a mammalian male accessory organ that develops under androgen stimulation. In rodents, prostate induction from the urogenital sinus (UGS) epithelium starts at embryonic day 17.5 (E17.5) in response to testicular androgens. UGS epithelial cells differentiate into solid cords that grow into the UGS mesenchyme. Each epithelial cord invades a distinct mesenchyme structure originating three lobes: ventral, dorsolateral and anterior. Thereafter prostate undergo morphological changes, such as stromal differentiation, epithelial branching and cavitation (canalization) during the first three weeks of prostate development (Vilamaior et al., 2006).

The ventral prostate (VP) epithelium branches early in postnatal life achieving ~50 tips in the first week and ~150 by the second week, which is approximately 80% of the tips present in a adult gland (Sugimura, 1986). It was reported that polarized fluid transfer rather than cell death is responsible for the lumen formation in prostate (Pearson et al., 2009). However, apoptosis does occur in VP epithelium together with the differentiation of secreting epithelial cells during canalization (Bruni-Cardoso and Carvalho, 2007), thus suggesting that lumen formation is a combination of both lipid transfer and cell deletion within the solid cords.

All morphological events are coupled interactions between epithelium and mesenchyme/stroma in space and time (Cunha, 1972; Cunha et al., 1983). In this manner, morphogens are of great importance for VP development (Prins and Putz, 2008). Some of these paracrine factors function as “andromedins”, androgen-induced ligands produced in the mesenchyme capable to induce prostate initiation. For instance, FGF10 is a candidate andromedin (Lu et al., 1999; Pu et al., 2007), but fails to induce prostate initiation *in vitro* from the FGF10 KO UGS, in the absence of testosterone (Donjacour et al., 2003).

CXCL12/SDF1, FGF10 and TGF-β are among the paracrine factors up regulated in prostate stromal cells, stimulated by the interaction with epithelial cells (Chambers et al., 2011). SDF1 is a chemokine that signals through the G protein-coupled receptor CXCR4. Extracellular matrix (ECM) contact modulates CXCR4 expression on prostate cancer cells (Kiss et al., 2013). The blockage of SDF1-CXCR4 signaling pathway with AMD3100 inhibits branching morphogenesis in the urothelic bud (UB) (Ueland et al., 2009), submandibular salivary gland (SMG) and pancreas, and also impairs lumen formation in pancreas (Hick et al., 2009).

Some of the paracrine factor with known function in VP development binds heparan sulfate (HS) with different affinities (Xu and Esko, 2014) and these interactions

require particular sulfation patterns (Ashikari-Hada et al., 2004). One amino acid change at the HS binding-site of FGF10 can turn it into FGF7-like in terms of HS binding and ECM diffusion, with reported impact in SMG and lacrimal gland epithelium branching *in vitro* (Makarenkova et al., 2009). Accordingly, different HS structures modulate FGF signaling to the SMG epithelium *in vitro* resulting in different branching architectures (Patel et al., 2008).

HS sulfation seems to be specifically regulated by changes in expression of the HS biosynthetic pathway (Lindahl et al., 1998), with special attention to the HS-sulfotranferases (responsible to decorate HS with sulfate), sulfatases (which removes sulfate groups) and Heparanase (capable to cleave HS chains at specific locations). Sodium chlorate inhibits the synthesis of 3'-Phosphoadenosine-5'-phosphosulfate (PAPS), which is a sulfate donor for the enzymatic sulfation reactions in the cell. Sodium chlorate inhibits protein (Baeuerle and Huttner, 1986) and glycosaminoglycans (GAG), in particular HS, sulfation (Safaiyan et al., 1999).

Induction of the prostate epithelial buds require highly sulfated heparan sulfate, which results from the activity of heparan Sulfate 6-O-sulfotransferase 1 (Hs6st1) expression and testosterone-dependent down regulation of Sulfatase-1 (Sulf1). This combination increases the ΔUA2S-GlcNS6S disaccharides content on HS chain at the male UGS epithelium. Sulf1 expression in the urogenital sinus epithelium inhibits bud formation (prostate induction) (Buresh et al., 2010; Buresh-Stiemke et al., 2012). However, the role of sulfation on postnatal prostate epithelial morphogenesis was yet to be solved.

So we hypothesized that HS sulfation plays an important role in epithelial morphogenesis, due to its property to fine-tune extracellular signaling. To test our hypothesis we used sodium chlorate in different concentrations in 3D cultures of a human non-tumor prostate epithelial cell line RWPE-1, and in *ex vivo* VP cultures. Our results indicate that acinar morphogenesis in 3D cell culture is highly sensitive to sulfation levels and that epithelial canalization and branching depend on different HS-sulfation pattern. SDF1 partially recovered canalization on the VP *in vitro* and acinar morphogenesis by RPWE-1 in 3D cell culture in a low sulfation environment, indicating that SDF-1 plays a role in epithelium morphogenesis and that its interaction with HS sulfation controls SDF1-CXCR4 signaling. Altogether our findings unveil a role for sulfation on VP epithelium canalization and branching morphogenesis in general and SDF1/CXCR4 signaling in particular.

Results

RWPE-1 cells synthesizes HS trough different HSPG on acinar morphogenesis

Human normal prostate epithelial cell line RWPE-1 in matrigel 3D cell culture mimics acinar morphogenesis (Tyson et al., 2007). Isolated cells from 2D culture added to 3D matrigel enviroment proliferate and organaizes smal aggragates within few days of culture,

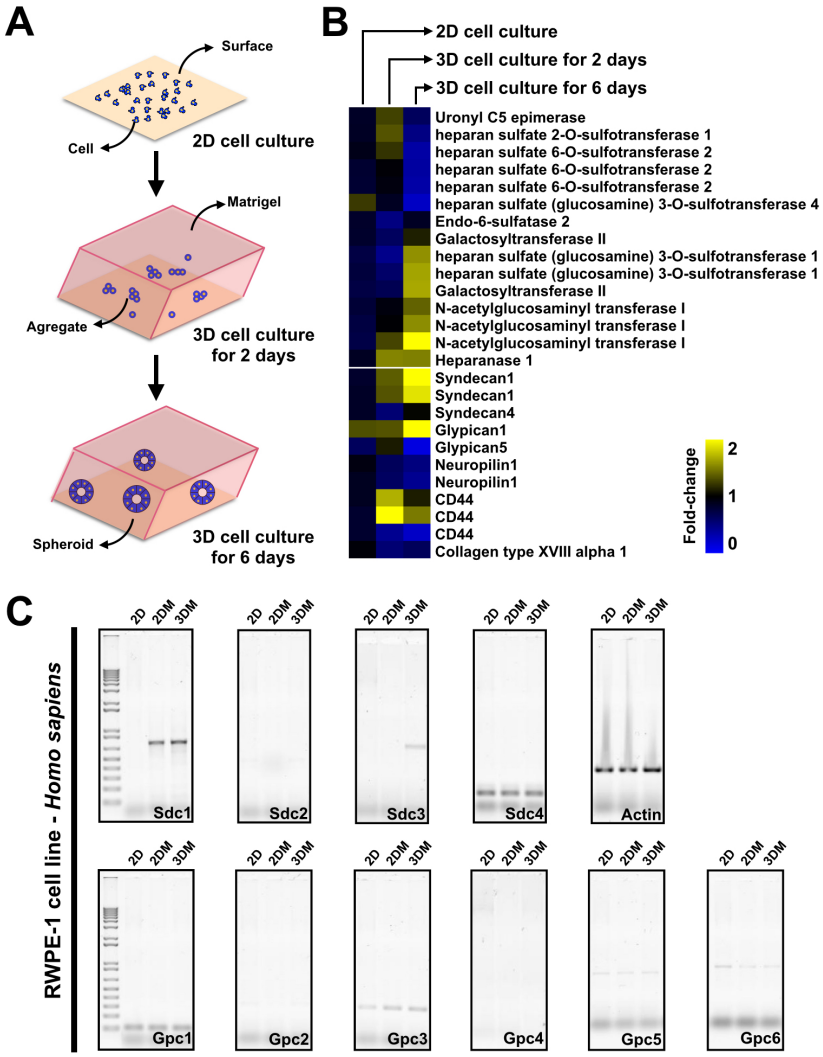

Fig 1. **RWPE-1 expresses HSPG throughout acinar morphogenesis.** (A) Human normal prostate epithelial cells, RWPE-1, grown in 2D over a flask substrate forms a cell monolayer sheet, when transferred to 3D in matrigel it takes 2-3 days to form aggregates of cell without a defined shape yet. Then it forms spheroids with lumen by day six. (B) Data mining on global expression analysis (GEO accession #: GSE30304) of this model shows differential expression of enzymes involved in HS biosynthesis pathway, in particular Hs2st1, Hs6st2, Hs3st1 and Hs3st1 and the HSPG, such as syndecan1, glypican5 and CD44 (C) RT-PCR of the syndecan1-4 and glypican1-6 RWPE-1 samples grown in 2D plastic substrate, 2D matrigel substrate and 3D in matrigel, confirms the expression of HSPG throughout acinar morphogenesis.

then by the end of a week, theses cells form spheroids, with basoapical polarization and lumen (Fig.1 A). Our data mining on a microarray database avaiable for this model showed differential expression of HS biosynthesis enzymes and HSPG (Fig.2 B) (Li et al., 2013). RWPE-1 cells down modulate Hs2st1, Hs6st2 and Hs3st4 expression, while it increases the expression of Hs3st1, Syndecan-1, Glypican-1 and CD44 when forming the spheroids structure on matrigel 3D culture. We validated the expression Syndecans and Glypicans through RT-PCR, which suggested that RWPE-1 cell expresses Syndecan-1, 3 and 4, and Glypican-1, 3, 5 and 6, albeit Syndecan-1 and 3 seems to be conditioned by matrigel and spheroid organization, respectively. All together suggests that HS plays a role in RWPE-1 acinar morphogenesis in 3D cell culture through different HSPG.

Sulfation reduction abolishes acinar morphogenesis in RWPE-1 3D cell culture

We decided to investigate whether sulfation would play a role in RWPE-1 acinar morphogenesis, because the cells differentially expresses HS sulfotrasnferases. So we made use of sodium chlorate to inhibit synthesis of PAPS, which donates sulfate for sulfotransferases reactions, including HS editing, such as HS 6-O and 2-O sulfation (Safaiyan et al., 1999). Concentrations of 20, 30 and 50 mM sodium chlorate treatment abolished sphereoids formation of RWPE-1 cells grown in matrigel 3D cell culture (Fig.2 A). In this experiments the average counts of shpherois por mm^2 were 16.9 on control group and it reduced to less than 2 on all chlorate concentrations. Thus it suggests that sulfation plays a roles in RWPE-1 acinar morphogenesis.

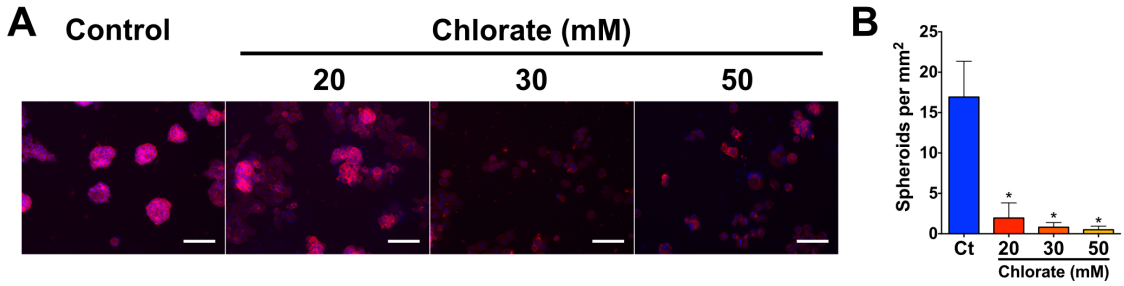

Fig 2. **Acinar morphogenesis in RWPE-1 3D cell culture is inhibited by chlorate treatment.** (A) RPWE-1 cells form spheroids after six days of culture in control group and chlorate impairs acinar morphogenesis at all concentration shown by the reduced (B) number of spheres. Scale bar: (A) 100 µm. Bars on column graphs represent s.d. of (B) mean of five fields; this experiment was performed in duplicate. Ordinary one-way ANOVA multi comparison test followed by Tukey's multiple comparisons test was performed (*) $p<0.05$.

Dose-dependent sulfation reduction impairs epithelial branching morphogenesis and canalization on ventral prostate development

Prostate epithelium expresses higher levels of Hs6st1 and lower levels of Sulf1 at early stages of morphogenesis. It results a higher content of 6-O sulfated HS and initial bud formation (Buresh et al., 2010; Buresh-Stiemke et al., 2012). We also used sodium chlorate on VP *in vitro* to understand the role of sulfation on ventral prostate (VP) epithelial morphogenesis (Fig. 3A). Chlorate treatment reduces the number of epithelial tips at 50 and 100 mM (Fig. 3B) and impairs lumen formation at 20, 50 and 100 mM (Fig. 3C). Because lumen formation is affected at lower chlorate concentration (20 mM) it suggests independent roles for sulfation on VP epithelial canalization and branching. Also VP treated with 50 mM chlorate differentially expressed Hpse1 and Mmp2 relative to control, but not Mmp9 (Fig. 3D), which correlates with higher MMP2 activity on culture medium (Fig. 3E), indicating difference in ECM remodeling on chlorate treated. Thus HS

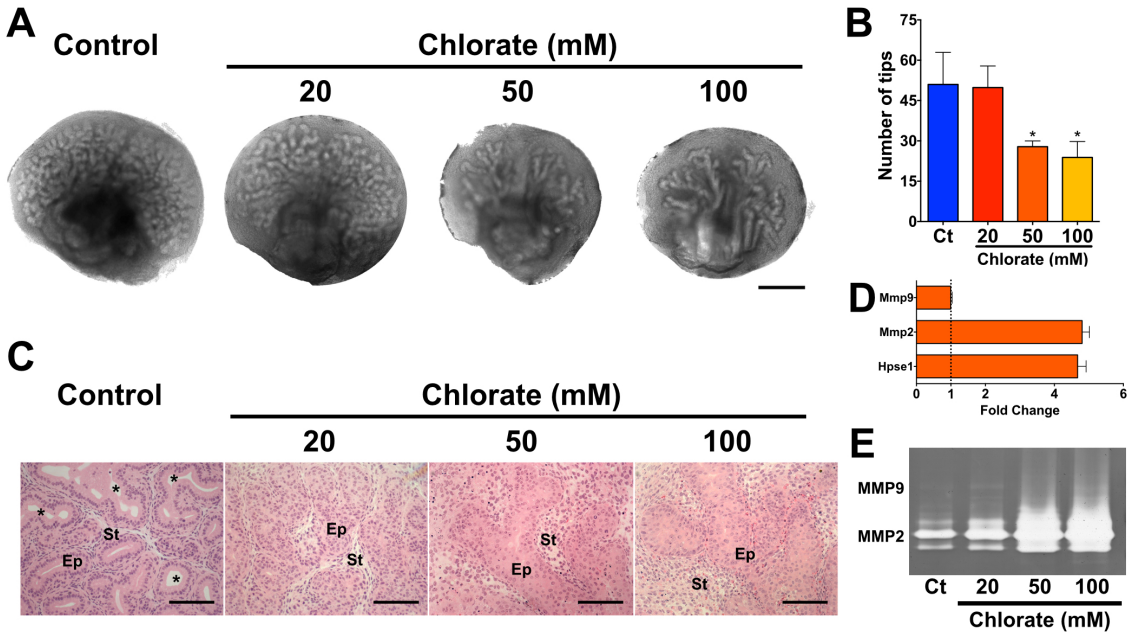

Fig 3. **VP epithelial canalization and branching inhibition on *in vitro* dose-dependent chlorate treatment.** (A) VP grown *in vitro* treated in a dose-dependent manner with sodium chlorate (20, 50 and 100 mM) reduced branching morphogenesis, shown by lower (B) number of epithelial tips, at concentrations 50 and 100 mM. (B) Hematoxilin & eosin stained sections of the VP epithelial tips. Cavitation results in ducts with simple columnar epithelial tissue and evident lumen (*). Chlorate treatment in all concentrations inhibited cavitation keeping a solid epithelial cord. (D) 50 mM chlorate treatment up regulated Hpse1 and Mmp2, but not Mmp9, mRNA expression compared to control (dash line) VP. (E) Gelatin zymography of culture medium at PND6 showing the increase of MMP2 on chlorate treated group at 50 and 100 mM. Scale bar: (A) 500 µm and (C) 50 µm. Bars on column graphs represent s.d. on (B) biological triplicates and (D) technical triplicate of a *pool*. Ordinary one-way ANOVA multi comparison test followed by Tukey's multiple comparisons test was performed (*) $p<0.05$.

sulfation reduction unveils independent roles for sulfation on epithelial branching and lumen morphogenesis during prostate development that may involve extracellular matrix turnover.

SDF1 induces lumen formation independently of HS sulfation

Sulfate reduction in HS interferes with different signaling pathways due to the binding properties of the morphogens (Xu and Esko, 2014). So we decide to test whether SDF1 (Fig. 4A and B), FGF10 and TGFβ1 (data not shown) could recover epithelial canalization and branching on chlorate treated VP. Prostate stromal cells differentially express these paracrine factors when in contact with epithelial cells (Chambers et al., 2011).

SDF1 impaired epithelial branching morphogenesis in combination with chlorate (Fig. 4A), however comparison of canalization among different concentrations of SDF-1 on 50 mM chlorate treated VP reveled that SDF-1 rescues lumen formation at the epithelial distal tips on low sulfated environment, as shown by the histological sections (Fig. 4B), albeit epithelium does not differentiate into simple columnar epithelial tissue as in a control VP (Fig. 3A). These results place a role for SDF1-CXCR4 pathway on VP epithelium canalization, even at low sulfated environment.

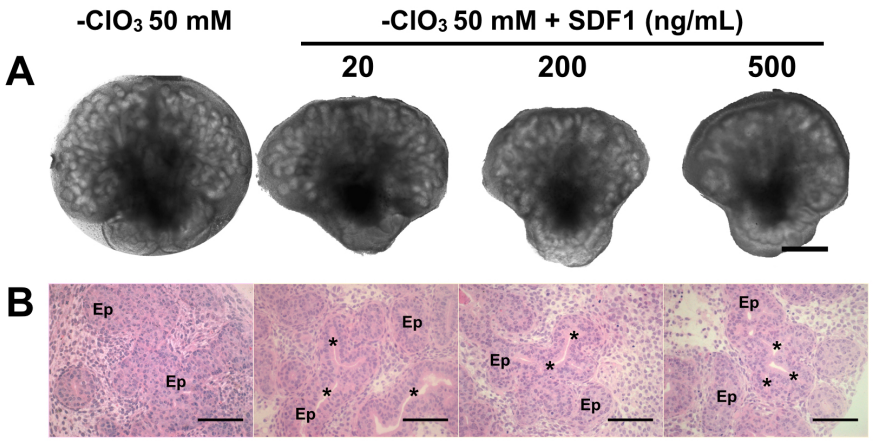

Fig 4. **SDF1 induce lumen formation on low sulfated environment.** (A) VP *in vitro* treated with 50 mM sodium chlorate was treated with increasing concentration of SDF1. (B) Histological sections stained with H&E of the epithelial tips shows epithelial lumen formation (*), compared to the chlorate treated group, even tough it is not composed of a single columnar epithelial tissue. Scale bar: (A) 500 μm and (B) 100 μm.

SDF1-CXCR4 signaling mediates RWPE-1 acinar morphogenesis

Because we observed that SDF1 could rescue lumen formation on VP treated with chlorate, we decided to investigate the role of SDF1-CXCR4 signaling on RWPE-1 acinar morphogenesis and whether this chemokine could rescue acinar morphogenesis on low sulfated environment.

So first we treated RWPE-1 cell on matrigel 3D culture with different concentrations of SDF-1 (Fig. 5A) that increased the average numbers of spheroids per mm^2 of 56.7 on control group to 101.7 on 500 ng/mL SDF-1 treated group (Fig. 5B) and the average diameter increased from 38.2 μm to values higher than 47.3 irrespective of SDF-1 concentration (Fig. 5C).

Then we inhibited SDF1-CXCR4 signaling with AMD3100 (Fig. 5D) which decreased the average number of spheroid formed per mm^2 of 40.6 on control group to an average of 11.5 in the 100 μM AMD3100 treated group (Fig.5 E). Signaling inhibition with 100 μM AMD3100 also reduced average spheroids diameter from 37.6 μm in the control group to 27.9 μm in treated groups (Fig.5 F).

Finally, we added SDF-1 to chlorate-treated RWPE-1 cells (Fig. 5G) and observed that the average number of spheroids per mm^2 increased from 9.5 in 20 mM sodium chlorate-treated to 21.3 in 200 ng/mL SDF-1 combined with chlorate treatment (Fig. 5H) as well as an increase on average diameter from 32.1 μm in the sodium chlorate-treated group to 39.4 μm in 200 ng/mL SDF-1 combined with sodium chlorate treatment (Fig. 5I).

These results suggest that SDF-1 signaling through CXCR4 plays a role in acinar morphogenesis contributing to the number of aggregated cells that organizes into spheroids. Sodium chlorate treated cells under stimulation with 20 and 200 ng/mL of SDF-1 form more spheroids than without SDF-1 stimulation with average diameter of 37 and 39.4 μm, similar to control conditions. However it was a lower signaling saturation point in the low sulfation environment, given that the highest stimulation was achieved on 200 ng/mL of SDF-1 different from control, which needed stimulation of 500 ng/mL SDF-1 to achieve its highest outcome.

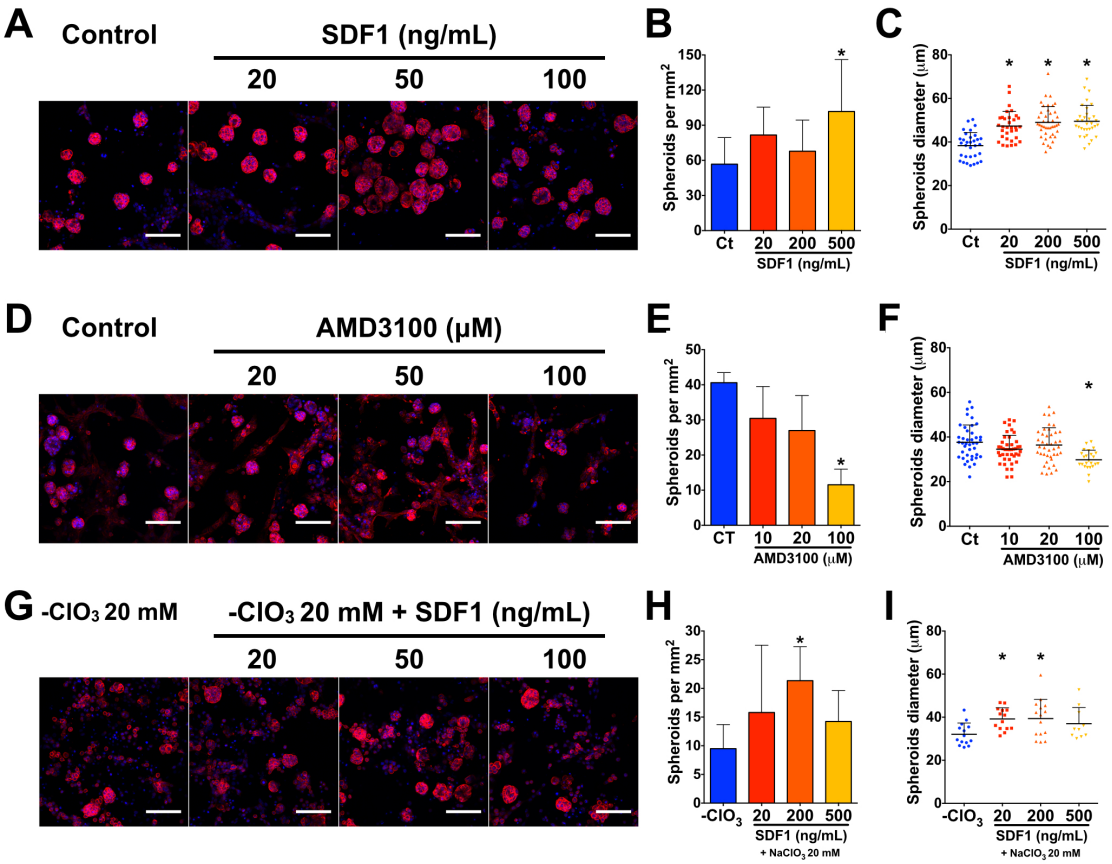

Fig 5. **SDF1-CXCR4 signaling pathway on acinar morphogenesis in RWPE-1 3D cell culture.** (A) RWPE-1 spheroids formed after six days of culture with increasing dose of SDF1, stained with phaloidin-TRITC and dapi, (B) resulted in a higher number of spheroids and (C) larger diameter. (D) When grown in the presence of AMD3100, (E) it reduces the number of spheroids and (F) reduces its diameter. (G) And SDF1 added to 20 mM chlorate treated cells resulted in rescue of (H) spheroids formation at a maximum on 200 ng/mL and (I) diameter as also. Scale bar: (A, D and G) 100 µm. Bars on graphs represent s.d. of the group. Two-way ANOVA multi comparison test followed by Tukey's multiple comparisons test was performed (*) $p<0.05$.

Discussion

Ventral prostate epithelial induction from the UGS epithelium requires HS (Buresh et al., 2010), so epithelial cords becomes enriched with tri-sulfated HS, but the roles of sulfation on postnatal prostate epithelial morphogenesis was yet to be know. In here we show that sulfation interferes on acinar morphogenesis of normal prostate epithelial cell RWPE-1 in 3D matrigel, where cells differentially express HS biosynthesis genes and HSPG, as they organize into spheroids.

VP epithelial canalization and branching demand on sulfation. Increasing concentrations of sodium chlorate gradually reduces 6-O and 2-O HS sulfation, due to inhibition of the synthesis of PAPS, a sulfate donor for HS sulfotransferase reactions (Safaiyan et al., 1999). Sodium chlorate treatment first inhibited epithelial canalization then epithelial branching in the VP in a dose-dependent manner, so different mechanisms should explain these distinct effects. Sodium chlorate also blocks initial epithelial buds formation in embryonic growth of the prostate (Buresh et al., 2010), reduces epithelial branching morphogenesis in lung (Izvolsky et al., 2003), and in kidney without interference in lumen formation (Steer et al., 2004).

One mM chlorate fully inhibits protein sulfation without any major change in expression pattern in PC12 cell line (Baeuerle and Huttner, 1986). In our hands, morphological changes in the epithelium happened in the range of 20-100 mM, indicating that is likely due to an effect on HS sulfation. Moreover, it is known that heparan sulfate afffects epithelial branching in other systems. Nsdt2 HS N-sulfation regulates FGF-Shp2 signaling cascade in lacrimal gland development (Pan et al., 2008). In mammary gland, epithelial branching is inhibited with the absence of HS in the MMTV conditional Ext1 KO mice, while inhibition of 2-O sulfation ablates side branching and end bud branching in MMTV conditional Hs2st1 KO mice (Garner et al., 2011). On the other hand conditional Ndst1 KO impairs lobuloalveolar extension resulting in less milk production (Crawford et al., 2010). In contrast, the combination of Ndst1 and Ndst2 KO results in reduction of epithelial growth and increase in primary and secondary branching (Bush et al., 2012). Urotheric bud (UB) branching requires HS 6-0 sulfation for proper growth factor binding (Shah et al., 2011), while 2-O sulfation controls growth factor binding regulators of early induction of metanephric mesenchyme (Shah et al., 2010). In the submandibular salivary gland different HS structures modulates epithelial branching outcome (Patel et al., 2008) and 3-O sulfation is involved in a positive loop to control KIT+ progenitor cells (Patel et al., 2014).

SDF1-induced partial recovery of canalization in sodium chlorate-treated VP likely reflects the HS effect on the local concentration of the ligand rather than a dependence on specific HS sulfation pattern to trigger signaling. This suggestion relies on the fact that SDF-1 binding to HS (Amara et al., 1999; Murphy et al., 2007) depends on 6-O sulfation (Uchimura et al., 2006), the major type of sulfation reaction affect by the treatment, and that HS increases local concentration of this chemokine at the cell surface without forming a tertiary complex with ligand-receptor (Kuschen et al., 1999). Furthermore, SDF1 induces lumen organization in pancreas (Hick et al., 2009) and epithelial branching in the kidney (Ueland et al., 2009).

RWPE-1 3D cell culture revealed that SDF-1 enhances, while AMD3100, an inhibitor of SDF1-CXCR4 signaling, compromises acinar morphogenesis. Then SDF-1 recovers acinar morphogenesis in the low sulfatation environment. The growth kinetics, however, achieves a saturation point within the concentration range used, based on spheroids counts and diameter measurement. Theses findings suggested that HS must control the local concentration of the chemokine, thus buffering the signal. Once sulfation is altered by chlorate and binding of HS is reduced, SDF-1 becomes free to signal without a proper HS buffering control, so it can saturate the receptor, that internalizes to the cell thus interrupting signaling (Minina et al., 2007; Signoret et al., 1997).

Although FGF10 and TGFβ1 had no effect on canalization in the low sulfation environment, it has been extensively reported that FGF10 signals duct elongation, epithelial branching and cell differentiation (Huang et al., 2005), albeit FGF10 alone does not recover *in vitro* prostate bud formation in the FGF10 KO UGS, and combined treatment with testosterone is necessary to induce branching (Donjacour et al., 2003). Testosterone is responsible for the down regulation of Sulf1 and accumulation of ΔUA2S-GlcNS6S disaccharides in the epithelium (Buresh et al., 2010), thus enabling FGF10 signaling mediated via FGFRiiib (Huang et al., 2005). Similar finding was reported for the SMG, in which HS 6-O-sulfation favors epithelial elongation and 2-O-sulfation, with either N- or 6-O-sulfation, promotes end bud cleft and enlargement (Patel et al., 2008).

Extracellular matrix turnover also impacts VP development. Epithelial branching was inhibited at 50 mM sodium chlorate, and the expression of both *Hpse1* and *Mmp2* was up regulated. Earlier studies reported the presence of MMP2 in the epithelium and surrounding stroma, while MMP9 was present at the growing tips (Bruni-Cardoso et al., 2008), and that *in vitro* silencing of *Mmp2* or inhibition of MMPs activity reduced VP growth and epithelial cell proliferation, with an associated accumulation of collagen fibers in the ECM (Bruni-Cardoso et al., 2010a; Bruni-Cardoso et al.,

2010b). More recently our group suggests that *Hpse* plays a role in early epithelial growth during the first postnatal week of VP development (accompanying manuscript). Yet, the mouse *Hpse* is expressed during SMG development stage and releases FGF10 from Perlecan HS to regulate epithelial growth and branching (Patel et al., 2007).

In conclusion, prostate epithelial morphogenesis involves HS/HSPG expression and sulfation. Epithelial canalization and branching morphogenesis might be regulated by distinct HS sulfation patterns, which remains to be elucidated. SDF-1 signaling triggers canalization (lumen formation), reverting the effect of sodium chlorate treatment which establishes a role for this paracrine factor on prostate epithelial morphogenesis, and suggests that HS buffers SDF-1 signaling, by controlling availability and local concentration at the cell surface. Our data also supports the importance of testosterone on VP *in vitro* growth, to keep sulfation at a steady level, otherwise HS-binding paracrine factor have their signaling outcome compromised. All together corroborates with the idea that HS perfect refinement of its ligands signaling, in space and time, results in proper VP development, as it is in other organs.

Material & Methods

Ventral prostate organ culture

Rat (Wistar) HanUnib:WH VPs were dissected with fine forceps under stereoscope at postnatal day (PND) 0 and placed over PTFE membranes (Millipore Corp., Bedford, MA) inside 24-well plates. Thus each well was filled with 500 µL with DMEM and Ham's F12 nutrient mixture at 1:1 ratio supplemented with 1% Insulin, Transferrin and Selenium (ITS) (Gibco, Grand Island, NY) and 10 nM testosterone cypionate (Novaquímica, São Bernardo do Campo, SP, Brazil). Sodium chlorate (Sigma-Aldrich, Saint Louis, MO, USA) and SDF-1 (PeproTech Brasil, Ribeirão Preto, SP, Brazil) were diluted in water and added to the medium at final concentration indicated in each experiment. Medium was changed and picture taken on inverted microscope AxionObserver every second day, and culture last for 6 days. The animal-handling and experimental protocols were approved by the University's Committee for Ethics in Animal Experimentation (Protocol no. 2920-1)

Histological sections

After culture VP were fixed in 4% paraformaldehyde (PFA), dehydrated in an increasing series of ethanol and embedded in HistoResin Mounting Media (Leica Biosystems, Wetzlar, Germany) for two hours and blocked. Then 2 µm sections were made with glass-knife and stained with hematoxilin & eosin. Finally images were taken in Zeiss Axioskop microscopy, and images were captured with a Zeiss Axiocam MRC CCD camera (Zeiss, Oberkochen, Germany).

Real-time PCR

Ilustra RNAspin Mini kit (GE Healthcare, USA) was used to isolate RNA from a VP pool (at least three per group) according to the manufacture. Then RNA was transcribed into cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA) according to manufacture. Finally proximally 20 ng of cDNA was used per well on a 20 µL reaction containing cDNA, distilled water, TaqMan 2x PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA) and Taqman inventoried assays. The assays used were for Hpse1 (Rn 00575080_m1), Mmp2 (Rn02532334_s1), Mmp9 (Rn00579162_m1), 2-microglobulin (internal control for *in vitro* VP) (Rn00560865_m1) and HPRT (internal control for dissected VP) (Rn01527840_m1). The reaction was performed on 7300 Real Time PCR System

(Applied Biosystem, Foster City, CA, USA) then ΔΔCt was computed and the results expressed as fold-change.

3D cell culture in matrigel

Normal human prostate epithelial cell line, RWPE-1 (ATCC® CRL-11609™), was grown on Keratinocyte Serum Free Medium (K-SFM) supplied with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) (Life Technologies). Cells were detached from flasks with 0.05% Trypsin - 0.53mM EDTA (Life Technologies) solution after confluence and added at 250 cells/μL to a mixture of Standard BD Matrigel Matrix (BD Biosciences, NJ, EUA) and complete medium at ratio 1:3. 60 μL of the final mixture was plated inside a Glass 8 Well Sterile CultureSlide (Gibco, Grand Island, NY, EUA) and the gel let polymerize for one hour at 37 ˚C inside the incubator. After 300 μL cell culture medium was added to each well. Sodium chlorate, SDF-1 and AMD3100 (Sigma-Aldrich) was used at indicated final concentration. Culture medium was changed every second day and after six days of culture, the cells were fixed in 2% PFA, permeabilized with PBS-T 0.02% and stained with Dapi and Phalloidin-TRITC (Sigma). The chambers divisions were removed and the slide mounted with Prolong. Finally the images were made on confocal microscopy Zeiss LSM780-NLO (Carl Zeiss AG, Germany).

Transcriptome analysis

A data set containing triplicate samples of RWPE-1 and LNCaP cells cultivated in 2D, 3D for two days and 3D for six days in matrigel (GEO accession #: GSE30304) (Li et al., 2013) was accessed for specific gene expression profile inquire using data related to RWPE-1. All described genes related to the HS biosynthesis pathway and HSPG were present at the analysis. MultiExperiment Viwer (MeV) (http://www.tm4.org/mev.html) was used for the ANOVA statistical analyses and heat map, which is an average representation of probes for the differentially expressed.

Gelatin Zymography

Culture medium from *in vitro* VP on chlorate experiment were collected, and 10 μL of each sample, under nonreducing condition, were loaded into a 10% SDS polyacrylamide gel containing 0,1% gelatin and electrophoresis was performed at 4 ˚C. After SDS was removed from gel with 2.5% Triton X-100 and the gel was incubated overnight on 50 mM Tris–HCl solution, pH 7.4, containing 1 M $CaCl_2$, 0.1 M NaCl and 0.03% sodium azide at 37 °C. And finally the gel was stained with Coomassie brilliant blue (0.5% dye in 20% methanol and 10% acetic acid).

Data and image analysis

Data and statistical analysis were performed on Prims 6 for Mac OS X (© 1994 – 2014 GraphPad Software, Inc. All rights reserved, La Jolla, CA, USA) and image processing and analysis were performed on ImageJ (Version 2.0.0-rc-31/1.49v, NIH, Bethesda, MD, USA).

Acknowledgements

The authors thank the National Institute of Photonics Applied to Cell Biology (INFABiC), for the outstanding work in microscopy technologies. This work was funded by FAPESP (Grant no. 2009／16159-6) and CNPq. G.O.B is supported by a fellowship from FAPESP (no. 2012／17657-0).

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NOTA: Heparan sulfate proteoglycans in epithelial morphogenesis and physiology: knock out of Syndecan- 1 using CRISPR-Cas9

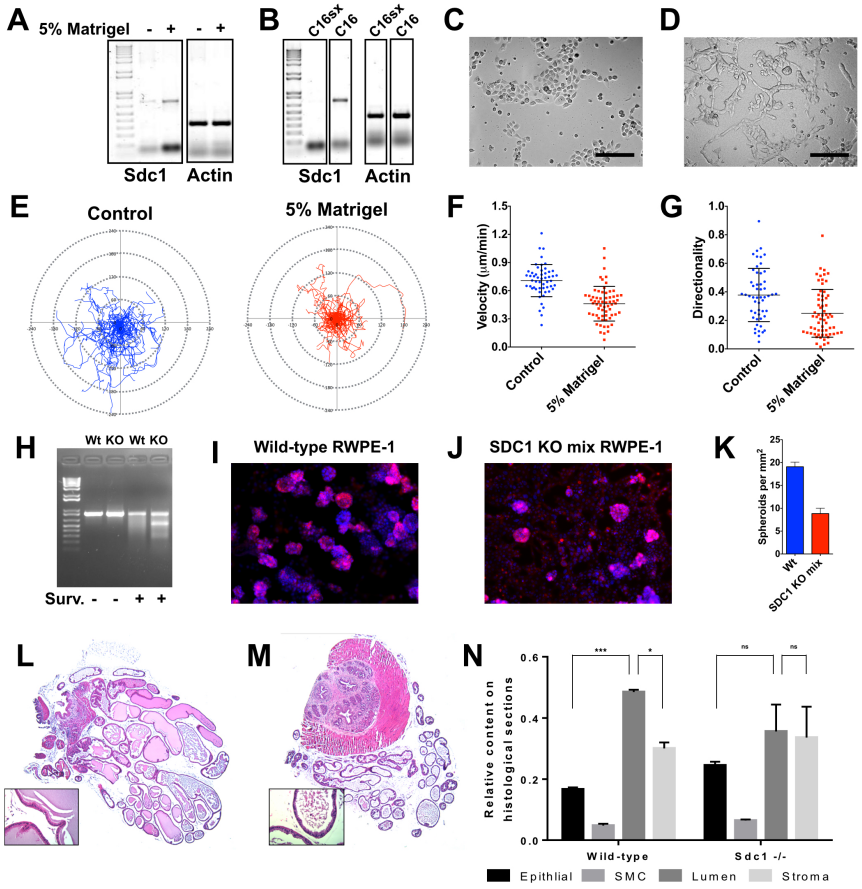

Figure 1. Laminin on basal membrane extracellular matrix induces Syndecan-1 expression on RWPE-1, which favors epithelial morphogenesis. Matrigel treatment induces RWPE-1 expression of Syndecan-1 on 2D cell culture (A). Laminin-1 peptide, C16, induces RWPE-1 expression of syndecan1 as well (B). RWPE-1 forms a monolayer sheet on hanging-drop culture (C), but 5% matrigel induce cells to organize into spheroids and cord-like structures (D). Cell migration of RWPE-1 was accessed on live cell imaging. Cells migrate towards all directions within a range of 180 µm, but 5% matrigel reduces cell migration range to 120 µm (E), velocity (F) and directionality (G). RWPE-1 cells were subjected to transfection of a CRISPR/Cas9 px456 plasmid design to target Syndecan-1. The mixed population was tested with the Surveyor assay and showed the presence of mutation in the sample (H). RPWE-1 wild-type (I) and Sdc1 -/- mixed population (J) of cells were grown in 3D with reduced-growth factor matrigel and the number of spheroids per field (K) was reduced in the Sdc1 -/- mixed population. 8-weeks wild-type (L) and Sdc1 -/- (M) mouse ventral prostate histology reveled rearrangement of compartments in the stereological analyses (N).

Manuscrito 3: Heparan sulfate fine-tunes prostate stromal-epithelial communication

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Several studies have proven the concerted and mutual communication between the epithelium and stroma, which determines the final organ architecture and function and goes awry in cancer. Deciphering the mechanisms involved in this communication is crucial to find new therapeutic measures. HS sequesters a number of secreted growth factors and cytokines, controlling their bioavailability to the target cells. This evidence suggests that HS is an important regulator of the extracellular matrix (ECM) and a key player in the cell-cell and cell-microenvironment communication during organs morphogenesis and physiology. In this work, we propose that by controlling HS biosynthesis and sulfation pattern, as well as the cleavage of the HS chain and/or the shedding of proteoglycans, prostate epithelial and stromal cells are able to precisely tune the availability of signaling molecules and modulate ligand-receptor interaction and intracellular signal transduction in order to regulate prostate homeostasis and physiology.

1. Introduction

Heparan sulfate (HS) is a major component of the extracellular matrix (ECM). It is synthesized in the Golgi apparatus by different enzymes working sequentially to determine the HS chain length and sulfation pattern [1-3]. After synthesis, the HS is exposed to the ECM covalently linked to proteoglycan core proteins, either attached to the plasma membrane or deposited in the surrounding space. HS functions as a reservoir impounding secreted growth factors and chemokines [4]. The presence, content and sulfation pattern of HS chains are controlled by both epithelial and stromal cells, hence regulating the bioavailability of signaling molecules [5].

Growth factors, chemokynes and cytokines are secreted molecules employed in autocrine and paracrine cell communication. FGF, TGFβ, Wnt, Shh and chemokines are involved in complex communications between prostate cells during different events [6]. FGF-7 and FGF-10 signaling regulates the morphogenesis of the prostate [7-9], submandibular salivary gland (SMG) [10] and other branched organs. FGF family members are also involved in the normal physiology of reproductive organs other than the prostate, contributing to epididymal function, and ultimately to sperm maturation [11].

On the other hand, the ECM plays pivotal roles in tissue structure, dynamics and cellular communication. The ECM is composed of soluble and insoluble components, which contribute to the tissue scaffold and to mechanical resistance; and acts as a reservoir of extracellular matrix signaling molecules. The matrisome project has revealed novel aspects of ECM composition [12], and its major contribution was an improved understanding of the ECM and its variations under different normal and disease states [13].

Herein we propose a *heparan sulfate tuning model* that places HS as an essential player in epithelial-stromal interactions that control organ development and

homeostasis. In this model, both epithelial and stromal cells determine (1) the expression of proteoglycan core proteins, (2) HS structure and content, (3) the secretion of extracellular sulfatases, the (4) production of sheddases and (5) HS-cleaving enzymes. Through the concerted regulation of these different steps, cells regulate HS structure, localization and mobilization from the matricellular space, and are endowed with the capacity to control local signaling, both in intensity and in efficiency, performing a duet under normal circumstances or turning into remarkable arias with tragic finales such as in cancer.

2. Heparan sulfate: synthesis and biology

Heparan sulfate biosynthesis takes place in the Golgi apparatus, where initially an amino sugar is O-linked to a serine residue of a core protein. Different enzymes such as N-acetylglucosamine transferase (GlcNAcT) and glucuronic acid transferase (GlcAT), N-acetylglucosamine N-Deacetylases/N-Sulfotransferase (NDST), glucuronyl C5-epimerase and O-Sulfotransferases work sequentially during HS synthesis (Figure 1). The different patterns of expression of these enzymes drive the unequal chain length and distribution of sulfate groups along the GAG chain in a variety of cells and tissues [14], although preserving a remarkably conserved domain distribution [15]. Interestingly, while NDST1 and NDST2 have similar effectiveness in the sulfotransferase and deacetylase activities, NDST4 is more effective in transferring sulfate groups than removing acetyl groups, and NDST3 shows the opposite behavior. Whereas NDST1 and NDST2 are strongly and ubiquitously expressed, NDST4 shows a remarkably restricted expression in the brain, heart kidney, lung, muscle and testis [16].

Sulfatases (Sulf1 and Sulf2) are enzymes that further process HS after its exposure to the extracellular space, removing O-sulfate groups. Reduction of HS sulfation levels has the potential to inhibit tumor growth [17, 18]. This trimming

reduces the capacity of HS to bind to different receptors [19, 20], modulate the accumulation of HS-binding growth factors in the ECM and cell surfaces [21], influence signal transduction via different receptors [22-24], and affect the expression and activity of HS cleaving enzymes, such as heparanase-1 (HPSE1) [25-27] (Figure 1).

As a general mechanism, HS-proteoglycans (HSPG) restrict the diffusion of growth-factor molecules, contributing to local cell signaling by establishing concentration gradients and preventing their degradation [28, 29]. The presence of HS throughout the tissues depends on HSPG expression [5]. Some HSPG are anchored to the plasma membrane by GPI-anchor family members or via a transmembrane domain, such as in glypican and syndecan, respectively. These HSPG can be solubilized via cleavage at specific domains by a group of enzymes, generally known as sheddases [30]. Shedding of the proteoglycan allows HS and its bound molecules to act in the matricellular space diffusively and not restricted to the attachment site [31]. The main sheddases are the Matrix Metalloproteinases (MMPs), such as MMP-9 -7 and -12 [25, 32] and the Disintegrin and Metalloprotease-domain-containing proteins (ADAMs) [33] (Fig. 1). Also GPI-linked proteoglycans, such as the glypicans, are released to the matricellular space through the action of enzymes such as Notum [34].

3. Heparan sulfate cleaving enzyme – Heparanase-1 (HPSE1)

Bioactive HS fragments are released from HSPG in mammals by cleavage of the GAG chain through the action of HPSE1 [35], which releases HS-bound growth factors. HPSE1 activity has been described in a number of tumors and is correlated with metastatic potential [25, 36-39] and angiogenesis [40-42].

HPSE1 is expressed as a 65 kDa protein and requires proteolytic processing in order to be activated as a 50 kDa/8 kDa heterodimeric enzyme [43, 44]. It is secreted

as a 65 kDa form and attaches to HSPG, which is internalized via a HSPG-dependent endocytosis pathway. Treatment with heparin blocks HPSE1 attachment to the HSPG, but not to the cell surface, indicating the existence of a possible HPSE1 receptor at the plasma membrane. Nonetheless, activation of the enzyme was observed under these conditions, suggesting the existence of a membrane surface processing [45]. HPSE1 intracellular processing occurs in the endosomal/lysosomal membrane in a pH-dependent manner by a membrane-bound enzyme or enzyme complex, likely involving cathepsin L [46].

HPSE1 overexpression in mice affected mammary-gland development, increasing side branching and alveolar formation, and also resulting in wider ducts [47]. Intriguingly, knockout mice for HPSE1 also showed increased side branching, although no difference was found in the width of the mammary gland ducts as compared to the wild type. Both phenotypes have a contribution from MMPs, due to their compensatory expression pattern in different tissues [48]. It is thus reasonable to assume that ECM remodeling with the aid of HPSE1 interferes with cell behavior, affects the turnover dynamics of different ECM components, and the expression of MMPs and their tissue inhibitors (TIMPs, RECK), with expected effects on morphogenesis, physiology, and probably cancer.

Branching morphogenesis of the SMG relies on HPSE1 activity, which is responsible for cleaving perlecan-HS, thereby releasing FGF10 that signals through ERK1/2 and promotes bud formation and epithelial branching [49]. Not only the SMG, but also lung, pancreas, kidney, prostate, and blood vessels undergo intense ECM remodeling during development. It seems possible that these organs depend on HS compartment remodeling in order to tune cell communication via the matricellular space.

Moreover, Heparanase-2, an HPSE1 homologue, has the potential to block HPSE1 activity due to its high affinity to HS and lack of enzymatic activity [50],

However, it has been shown, that the expression of HPSE2 is correlated to the severity of the lesion in squamous neoplasia of the cervix [51].

Given the elevated expression of HPSE1 in different cancers, which has been associated with invasiveness potential [32, 33] [52], many therapies targeting HPSE1 have been suggested for the treatment of cancer [39]. PI-88 is a highly sulfated mannose oligosaccharide that has shown some success in the treatment of hepatocellular carcinoma [53], and has been associated with docetaxel, an anti-mitotic chemotherapy drug, to reduce castrated-resistance prostate-cancer cells [54]. Defibrotide is an oligonucleotide used in combination therapies for the treatment of myeloma, with reported effects on HPSE1 inhibition [55].

4. **Heparan sulfate and signal transduction**

HSPGs make two main contributions in cells, via surface receptors. First, they interact physically with the extracellular signal molecules, and second, they modulate mechanical forces that cluster proteoglycans, leading to intracellular transduction.

Ligand-receptor signal transduction activation is modulated by HS in a variety of ways. For instance, Fibroblast Growth Factor Receptors (FGFR) have an extracellular domain that binds to HS [20], which is the basis for the FGFR activation in the absence of the ligand [56].

On the other hand, Transforming Growth Factor β-1 (TGFβ-1) responsiveness may be modulated by HSPG. TGFβ receptors (TβR) enter the cell via either clathrin-coated vesicles or caveolae, and these routes drive the receptor to internal cell signaling or rapid degradation of the receptor, respectively, depending on the ratio between TβR I and TβR II in the complexes formed between them. HS favors the formation of complex II, in which TβR I predominates over TβR II, enters calveolae, and is targeted to degradation pathways. HS-biosynthesis-defective cells respond

better to TGFβ-1 than do wild-type cells, and also have a higher ratio of ligand bound to TβR II/ TβR II, decreasing TGFβ-1 degradation [57].

Some studies have examined a cell lineage derived from human plasma-cell leukemia (ARH-77), which does not bind to type I collagen and has low amounts of HS on the cell surface, conferring a high potential to invade tissues. When these cells are induced to express syndecan-2 and 4, they acquire type I collagen-binding capacity and lose their invasiveness; but when induced to express glypican-1, a GPI-attached proteoglycan, they do not bind to collagen nor do they have the potential to invade, showing that although they increase the amount of HS on the cell surface, these different proteoglycans play distinct roles in cell motility and capability to bind ECM [58]. Furthermore, syndecan-4 (S4) has been shown to be a regulator of Rac1 localization in the cell leading edge in a PKCα-dependent manner, contributing to a directional migration sensitive to ECM clues [59]. The proposed mechanism dictates that clustering of S4 connects the extracellular matrix to the molecular mechanism to control cell motility [60]. Notably, HPSE1 may also play a role in syndecan clustering by interacting with the HS in a non-enzymatic way, and this also leads to increased cell motility [61]

In summary, heparan sulfate in the matricellular compartment regulates cell behavior dynamics by controlling (i) ligand storage and gradient formation, (ii) signaling pathway activation, (iii) cell-cell and cell-ECM interactions, and (iv) cell motility and migration, with remarkable effects on organ morphogenesis, physiology, angiogenesis, tumor progression, and metastasis.

5. Heparan sulfate tuning model – a prostate perspective

We propose that by modulating the production and assembly of the HS at the ECM, cell-cell communication is precisely tuned in two major steps: (1) defining the features of the signal reservoir (and its ability to interact and sequester the

extracellular signaling molecules), and (2) determining the signal release form (bound to HS fragments or to HSPG), eventually defining the downstream signaling pathway. This model also includes the possibility that stored factors are mobilized by either communicating cell under different physiological conditions (Fig. 2).

Extracellular Sulfatase1 (Sulf1) function in prostate development is a clear evidence of the tissue's capacity to modify the ECM composition and the resulting cell fate. Sulf1 is a 6-O endosulfatase, which removes sulfate groups from the heparan sulfate (HS) chains and is down-regulated at embryonic day 18 by androgens in the mice UGM to promote prostate development in males, but not in females. Sulf1 down-regulation promotes an increase in ΔUA2S-GlcNS6S HS disaccharide content in UGS mesenchyme and allows UGS epithelium budding [62]. In females, the presence of Sulf1 in the UGM and the resulting lower content of trisulfated HS disaccharides laed to a reduced activation of the FGFR and its downstream components ERK1/2 [62]. These data reveal the intrinsic relationship between the extracellular HS compartment and modulation of cell behavior. This role of Sulf1 in prostate induction reveals the importance of HS and HSPG and defines the matricellular compartment as an important part of the stages of development, homeostasis and disorders of the prostate.

Stromal regulation of epithelial cell behavior during prostate development and normal physiology has been further dissected to the transcriptional level. Gene-expression profiling of primary prostate epithelial cells in 3D cultures in the presence or absence of stromal cells revealed that the majority of genes induced by co-culturing stromal cells were cell adhesion, phophatidylinositol signaling, epithelial junctions, TGF-β and MAPK signaling pathway ontologies [63]. On the other hand, the epithelium affects stromal cells by producing soluble factors such as CXCL12, TGFβ, FGF, HGF and BMP [64]. These molecules are paracrine factors involved in the communication between epithelial and stromal cells [65].

Moreover, the transcriptome of aging prostate stroma showed the up-regulation of different chemokines, including CXCL12 (SDF-1), an HS binding ligand [66, 67], which is a pro-inflammatory factor that contributes to the development of BPH and lower-urinary-tract symptoms [68]. Nevertheless, prostate-tumor epithelial cells down-regulate the expression of TGF-β in fibroblasts, reducing ECM production and increasing cell motility [69]. It is evident that paracrine communication between cells is affected by extracellular molecules and is adjusted to promote tissue responses, both in health and in disease.

HS seems to be involved in adult prostate-gland biology as well. It is known that the prostate shrinks during the reproductive cycle in seasonal breeders and after castration, due to a series of events such as epithelial cell death [70], secretion reduction [71], and ECM remodeling [72-74]. The HS compartment is one of the ECM components in the rat ventral prostate (VP) that is affected by androgen deprivation. We found a reduction in the total amount of HS, an increase in HPSE1 expression and activation, and a switch from a predominant expression in the epithelium to the stroma after castration [75]. Interestingly, we have recently shown that HPSE-1 expression is inhibited in the VP of rats neonatally exposed to a high dose of 17β-estradiol, and that this inhibition occurred at the transcription level and was due to hyper-methylation of the gene promoter [76].

Thus, we propose an HS tuning model in which the communicating cells interact with each other not only by expressing the signaling molecules and their cognate receptors, but also by assembling the matricellular environment where a number of signals are stored and mobilized in different forms, resulting in variable bioavailability and hence affecting downstream signaling pathways (Fig. 2).

In summary, this model sets a new layer of complexity in the paracrine (and autocrine) communication between cells. The simple production of signal molecules aiming to a target receptor is challenged by the tuning effect exerted by the HS compartment. This means that, to really understand the interaction between cells, it

is mandatory to consider the role of HS in the matricellular space and the four steps that clearly affect it: HS biosynthesis, change in extracellular sulfation pattern, HS cleavage, and HSPG shedding. By considering these aspects of cell communication, we will be able to envision targets for drug development in order to treat disorders, which clearly reflects the establishment of new parameters in the communication among cells within an organ.

6. Limitations and perspectives

Other molecules contribute to the ECM function in coordinating cell behavior. Integrins are extraordinary receptors transducing inside-out and outside-in messages [77]. Though lacking high charge density, chain flexibility promoted by iduronic acid endows dermatan sulfate with growth factor-binding capacity [78]. Nonetheless, the ECM components themselves are sources of cryptic factors exposed upon proteolytic processing and interaction with specific receptors [79, 80]. Each of these aspect multifactores program of gene expression regulation by the ECM [81].

Although the above discussion is centered on HS and some aspects are inherent to more-comprehensive prepositions, the present model includes the notion that communicating cells play complementary and alternating roles in defining the ECM capability to retain signaling molecules and mobilize them, which in turn define the manner in which the signaling molecule reaches the receiver. By adding new layers of complexity in the stromal-epithelial communication, we propose the existence of new targets for intelligent drug design and new therapeutic approaches to many diseases.

7. Acknowledgments

Founding from FAPESP and CNPq is gratefully acknowledged. Guilherme Oliveira Barbosa was the recipient of CAPES fellowship.

8. Reference

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9. Figures

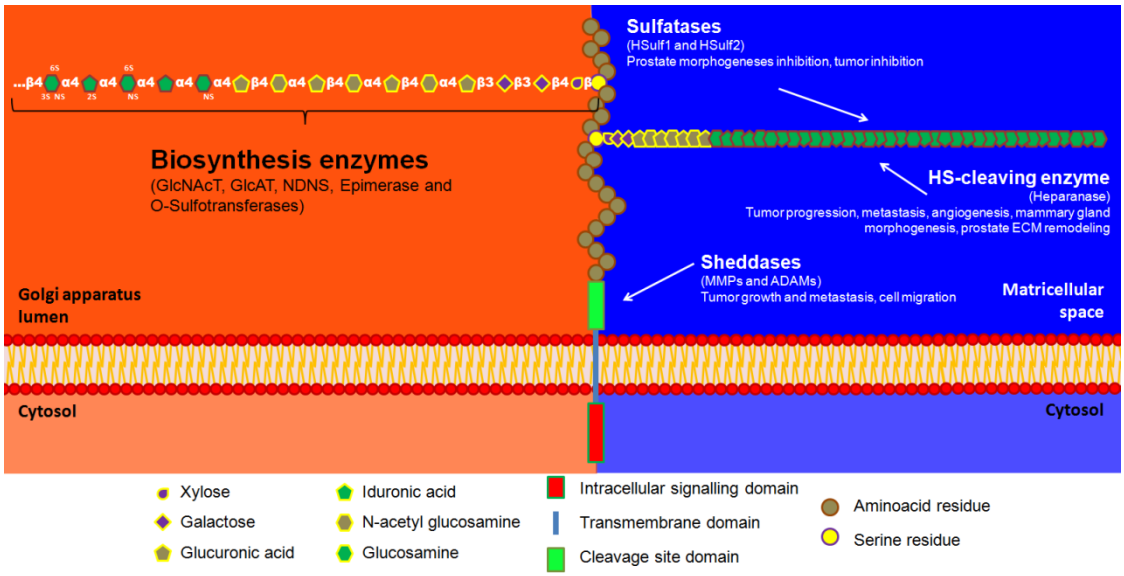

Figure 1. Some important factors that define the HS content and structure and the ability to bind signaling molecules in the ECM: biosynthesis enzymes (in the Golgi apparatus) and sulfatases (in the extracellular space); Sheddases release HSPG from the cell membrane, and HPSE1 cleaves HS fragments from the core protein and releases bound bioactive factors.

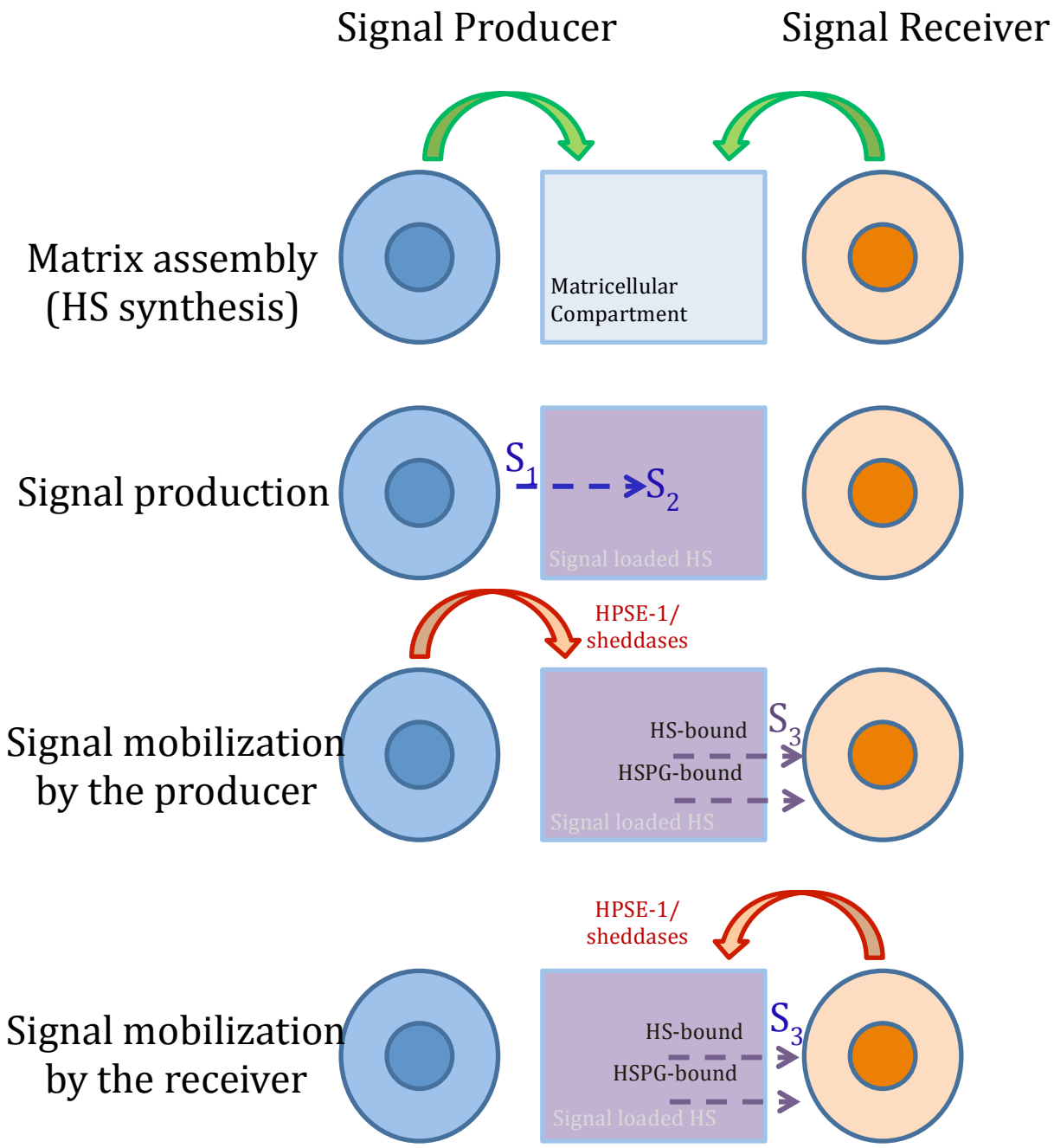

Figure 2. Heparan sulfate tuning model. Stromal-epithelial communication involves the production and secretion of molecules by the signaling cell (Signal producer) and the expression of the cognate receptors by the receiver (Signal Receiver). These signals can be soluble factors that do not bind to ECM and flow uni- or bidirectionally. The other class comprises signaling factors (blue arrow) that interact with HS and other components of the cell surface and the ECM (designated the matricellular compartment), collectively assembled by both cells (green arrows). Once bound to HS, either signal-producer or signal-receiver cells might mobilize HS-bound factors through ECM remodeling (orange arrows). Thus, a factor S1, produced by Cell A, remains in an HS-bound state (S2) in the matricellular space and is mobilized by HPSE1 or sheddases, to produce soluble S3 forms which are attached to HS fragments or to solubilized HSPG, respectively.

Discussão da tese

O heparam sulfato (HS) regula as sinalizações parácrinas entre epitélio e estroma no espaço e no tempo, contribuindo na regulação fina das diferentes etapas do desenvolvimento prostático. A determinação do padrão de sulfatação do HS, mediado pela queda de expressão de Sulfatase-1 regulada por andrógenos na indução prostática (BURESH et al., 2010), a expressão de Heparanase-1 pelo epitélio durante a primeira semana de desenvolvimento pós-natal (Manuscrito 1) e a manutenção da sulfatação e atuação SDF-1 na morfogênese epitelial, em particular na ramificação ductal, nesta primeira semana de desenvolvimento pós-natal (Manuscrito 2) (BURESH-STIEMKE et al., 2012) resultam na arquitetura associada ao funcionamento particular do órgão.

Neste sentido esse trabalho apresenta evidências de que o eixo heparam sulfato/Heparanase-1 possui papeis importantes no desenvolvimento pós-natal da próstata ventral de ratos, sugerindo que a edição do HS é um elemento fundamental da regulação da morfogênese epitelial de próstata. O desenvolvimento deste trabalho e os resultados obtidos, permitem estabelecer novas perguntas relacionadas aos diferentes mecanismos da interação epitélio-estroma e, em particular da regulação parácrina no desenvolvimento da próstata.

“Quais fatores de sinalização parácrina exercem papel de andromedina?” Esta é, talvez, a principal pergunta sobre a biologia do desenvolvimento da próstata. Este fator deve ser regulado por andrógeno nas células do mesênquima, provocar a indução prostática ao atuar no epitélio do seio urogenital, e, por definição, ser capaz de fazê-lo na ausência de testosterona. O FGF10, que é ligante de HS (ASHIKARI-HADA et al., 2004), atua na indução prostática e no desenvolvimento pós-natal de próstata ventral de roedores (HUANG et al., 2005; PU et al., 2007; THOMSON; CUNHA, 1999; TOMLINSON; GRINDLEY; THOMSON, 2004), mas não é capaz de promover a indução prostática na ausência de testosterona a partir do seio urogenital de camundongo mutado no gene *Fgf10* (DONJACOUR; THOMSON; CUNHA, 2003). Esta observação o desqualifica como candidato único ao papel de andromedina.

No entanto sabemos que a expressão de *Fgf10* é regulada por testosterona, ao passo que a expressão de *Bmp4* é inibida pelo andrógeno por vias independentes de FGF10 (HUANG et al., 2005; PU et al., 2007). BMP4 é um inibidor de indução

prostática e da ramificação epitelial durante o desenvolvimento da próstata, e sua expressão na região do seio urogenital decai após o primeiro pico de testosterona (LAMM et al., 2001). Na próstata, a ação desses dois fatores (FGF10 e BMP4) têm papeis opostos na ramificação epitelial, assim como ocorre no desenvolvimento do pulmão (WEAVER; DUNN; HOGAN, 2000).

As andromedinas podem ser fatores que levam à indução prostática e são positivamente regulados por andrógenos, mas também podem ser fatores inibitórios da indução prostática ("anti-andromedina"), regulados negativamente por andrógenos. Seria mais provável acreditar na combinação destas duas possibilidades, como já sugerido (THOMSON, 2008). No entanto, se as andromedinas são fatores de sinalização parácrinos ligantes de HS, a manutenção a homeostase do HS, através da regulação das enzimas responsáveis pela extensão da cadeia e definição do padrão de sulfatação (sulfotransferases, deacetilaces-sulfotrasferases e sulfatases), seria essencial para a correta definição de gradientes de difusão e correta determinação da biodisponibilidade destas andromedinas candidatas tanto na indução prostática como nos eventos relacionados à morfogênese ductal.

O estímulo androgênico regula negativamente a expressão de Sulfatase-1 no seio urogenital (BURESH et al., 2010). Este estímulo deve se dar via mesênquima, já que o epitélio do seio urogenital carece de receptor de andrógeno funcional (COOKE; YOUNG; CUNHA, 1991). Já a BMP4 modula positivamente a expressão de Sulfatase-1 no seio urogenital, resultando em diminuição do conteúdo de HS tri-sulfatado.

Com esses atores escalado, é possível especular que a testosterona modula inversamente a expressão de BMP4 e de FGF10 que, em conjunto, determinam a expressão das principais enzimas reguladores do padrão de sulfatação no HS no epitélio do seio urogenital. Este padrão específico, caracterizado principalmente pela predomínio de dissacarídeos trissulfatados, promoveria a sinalização mediada por FGF10. Isso poderia explicar a razão pela qual FGF10 não resgata a indução prostática no seio urogenital do animal com *Fgf10* mutado, já que a expressão de *Bmp4* não deve decair sem a presença de testosterona mantendo a expressão de Sulfatase-1 constante, da mesma forma que no seio urogenital de fêmeas, o que resulta no menor conteúdo de HS tri-sulfatado com impacto direto na sinalização de FGF10.

Para garantir o posto de andromedina do FGF10 e de "anti-andromedina" de BMP4, FGF10 poderia ser utilizado combinação com Noggin em cultura de seio urogenital. Este inibe a sinalização de BMP4 (ZIMMERMAN; DE JESÚS-ESCOBAR;

HARLAND, 1996) e tem participação na indução prostática (COOK et al., 2007). Dessa forma Noggin seria o agente responsável por induzir a homeostase de HS, via inibição de BMP4 e queda na expressão de Sulfatase-1, permitindo que FGF10 exerça seu papel de andromedina. Este racional poderia levar também à identificação de outros candidatos atuando nessas mesmas circunstâncias.

Neste trabalho não conseguimos mostrar nenhum mecanismo que explicasse a inibição de ramificação do epitélio prostático mediante o tratamento de clorato, que reduz o nível de sulfatação das células por inibir a síntese de PAPS, em cultura de próstata ventral. No entanto existe um modelo que combina a participação de FGF10 e BMP4 que se encaixa às ideias a cima propostas.

Inicialmente há expressão dispersa de FGF10 e BPM4 pelo mesênquima.

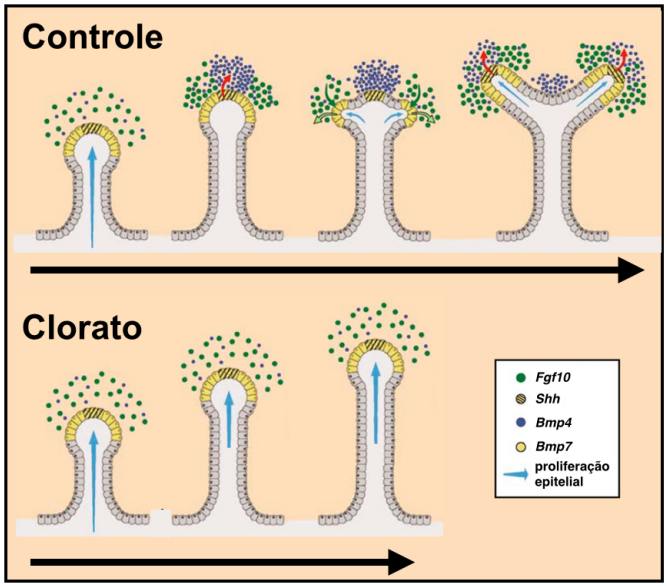

Figura 4. Modelo esquemático comparando o crescimento e ramificação do epitélio mediado por FGF10 e BMP4 numa condição controle cuja distribuição correta dos fatores parácrinos resultam em ramificação do epitélio, comparado com a situação clorato onde a sulfatação é reduzida, e a distribuição desses fatores, que são ligantes de HS, deve ser desorganizada, resultando em crescimento do epitélio sem ramificação. Esquema adaptado de (HUANG et al., 2005)

Com o avanço de crescimento das estruturas epiteliais, no centro do *tip* há uma queda na expressão de FGF10 e aumento de BMP4, o que criaria dois pólos de crescimento mediado por FGF10, originando os dois novos ramos do epitélio (HUANG et al., 2005). Agora podemos especular que no local onde a expressão de FGF10 cai e BMP4 aumenta, deve haver uma indução local de Sulfatase-1 que diminui localmente o nível de sulfatação de HS no epitélio e então promove a distribuição lateral de FGF10 para os locais onde o HS permanece sulfatado. Assim sendo, ao afetarmos a sulfatação com clorato, devemos estar impactando diretamente polarização na de FGF10 no tip em crescimento, de tal modo que a presença de FGF10 e BMP4 seja similar aos estágios iniciais de formação de brotos epiteliais a partir do epitélio do seio urogenital, causando o alongamento da estrutura epitelial com marcante redução na ramificação (Fig. 4).

A Heparanase-1 é a única enzima conhecida no genoma de vertebrados capaz clivar HS, participando ativamente da sua homeostase. Neste trabalho constatamos que sua expressão decai durante a primeira semana de desenvolvimento pós-natal. Tanto o tratamento com heparina como o silenciamento da expressão de Hpse-1, resulta no comprometimento do crescimento do epitélio no inicio da primeira semana pós-natal, que coincide exatamente com o período em que o crescimento epitelial dependente de sinalização via ERK1/2 (KUSLAK; MARKER, 2007). Assim sendo, a Heparanase-1 expressa pelo epitélio parece atuar na disponibilização de fatores parácrinos cuja sinalização depende de fosforilação de ERK1/2, juntamente com a liberação de fragmentos bioativos de heparam sulfato, assim como descrito do desenvolvimento da glândula salivar (PATEL et al., 2007). No entanto é notório que a fosforilação de ERK1/2 parece ser mais evidente no estroma, tanto no estágio de indução prostática, como na etapa de ramificação ductal. Essa sinalização no estroma parece ser acionada por Fator Neurotrófico Derivado de Linhagem Glial (GDNF), responsável por estimular a proliferação das células estromais (PARK; BOLTON, 2015). Com estas informações, podemos especular que a proliferação das células epiteliais seja dependente da proliferação e diferenciação das células estromais, que por sua vez depende da ativação de ERK1/2 ativadas por fatores solubilizados produtos da ação da Heparanase-1, em particular nas interfaces entre

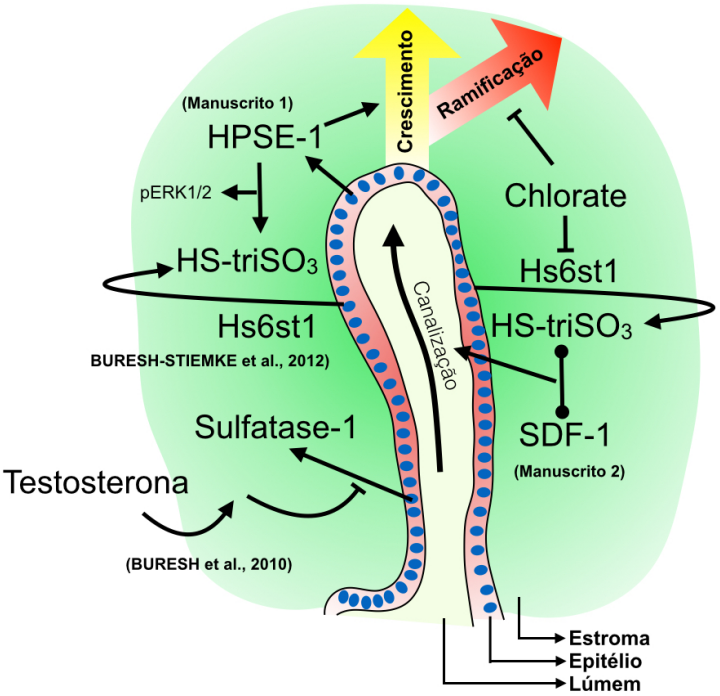

Figura 5. Modelo representativo dos achados nesse trabalho mostra que a ação da testosterona no mesênquima leva a redução de expressão de Sulfatase-1 no epitélio do seio urogenital, que por sua vez expressa Hs6st1, resultando no acumulo de HS trisulfatado. A Heparanase-1 que cliva HS, tem papel no crescimento e sua atividade esta relacionada sinalização ERK1/2 estimulada no estroma. O tratamento de clorato inibe sulfatação nas células, incluindo a de HS, por reduzir atividade de sulfotransferases com a inibição da síntese de PAPS, que resulta na inibição de ramificação e canalização. Já a sinalização por SDF-1 regula a canalização ductal.

epitélio e estroma, dada a localização predominantemente epitelial desta enzima.

As informações listadas acima, permitem sugerir que a homeostase do HS resultante do estímulo androgênico é responsável por definir o recrutamento,

estabelecimento de gradientes e concentração localizada dos fatores de sinalização parácrina que determinam o padrão de crescimento, ramificação ductal e canalização do epitélio em desenvolvimento, definindo a arquitetura do órgão.

Em conclusão, o papel da Heparanase-1 é regular o mecanismo a cima por clivar o HS e liberar os fatores reguladores do compartimento epitelial. Sua ação tem maior impacto no crescimento inicial do epitélio durante a primeira semana de desenvolvimento pós-natal. A canalização e a ramificação do epitélio são também dependentes de sulfatação, incluindo a sulfatação do HS, dado o comprometimento destes processos pelo tratamento com clorato. Por fim, SDF-1, que também é ligante de HS, é um indutor da canalização epitelial, sendo capaz de reverter o efeito de clorato na inibição deste processo (Fig 5). Embora não fique clara a razão pela qual este fator seja capaz de influenciar a morfogênese na presença de clorato de sódio, o que não aconteceu com a adição de TGF-b ou FGF-10, é possível que isto se ocorra em função do tipo de interação e dependência de HS e/ou de HSPG para interação com os respectivos receptores ou em função da existência de sequências temporais na sinalização destes diferentes fatores, nenhum dos quais pode ser revertido pela presença dos fatores isoladamente e em forma solúvel.

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Anexos

Material e métodos da tese

Dissecção de próstata ventral de rato neotano

As PVs foram coletadas no dia 0 pós nascimento para as culturas de órgão ou em diferentes datas, indicadas nos experimentos. Os animais, da linhagem HanUnib: WH (Wistar), foram adquiridos no Centro Multidisciplinar de Investigação Biológica (CEMIB - UNICAMP). Os procedimentos utilizados nesse trabalho foram autorizados pelo Comite de Ética no Uso de Animais do Instituto de Biologia da UNICAMP (Protocolo #. 2920-1).

Os animais foram sacrificados por decapitação e as PV microdissectadas. Para isso, usamos duas pinças para abrir a pele da região abdominal, bem como o peritônio. Uma vez acessado a região intraperitoneal, observando através de um estereoscópico e com o uso de uma microtesoura e de uma micropinça, fizemos a remoção da bexiga e do seio urogenital e glândulas associadas de todos os animais armazenando os em meio de cultura ou PBS. Assim que as estruturas de todos os animais foram removidas, passamos a dissecar a PV com duas micropinças nº 5, destinando-as aos próximos experimentos.

Cultura de órgão

- Placa de 24 poços (Cat # 92024, TPP, Trasadingen, Suiça)
- Membrana branca FHLC 0,45µm (Cat # FHLC01300, Millipore, Irlanda)
- Meio DMEM (Cat # D1145, Sigma-Aldrich Co., Saint Louis, MO, EUA)
- Meio HAM F12 (Cat # 00082 Vitrocell, Campinas, SP, Brasil)
- Ciprionato de Testosterona (Deposteron, Sigma pharma, Hortolândia, SP, Brasil)
- Insulina, transferrina, selênio (Cat # 41400-045, Gibco, Grand Island, NY, EUA)

As PV dissecadas no dia do nascimento do rato foram colocadas em membranas FHLC flutuando sobre meio de cultura (DMEM/HAM F12 1:1, suplementados com ITS 1x, e ciprionato de testosterona 10 nM). O meio foi trocado de dois em dois dias e foram feitas imagens em microscópio invertido, para que fosse processado o tamanha da área do epitélio. Para cada experimento um conjunto de tratamento diferente foi aplicado de acordo com o descrito nos resultados. Ao termino da cultura seis dias as PV foram encaminhadas para futuros processamentos (ex: Histologia e extração de RNA total)

Cultura celular 3D de linhagem RWPE-1 em MATRIGEL

- Cultura de célula epitelial prostática da linhagem RWPE-1
- Tripsina/EDTA
- Meio Keratinocyte-SFM, suplementado com EGF e Extrato de glândula pituitária (Cat # 17004-042, Gibco, Grand Island, NY, EUA)
- *Falcon*
-*Glass 8 Well Sterile CultureSlide* com *Polystyrene Vessel Lid* e *Safety Removal Tool, 1.2mL Volume* (Cat # BD 354108, BD Biosciences, NJ, EUA)
- Standard BD Matrigel Matrix (Cat # 356234, BD Biosciences, NJ, EUA)
- Dapi
- Faloidina-TRITC (Cat # P1951, Sigma-Aldrich Co., Saint Louis, MO, EUA)
- Microscópio AxioObserver confocal LMS780 (Zeiss, Germany)

As células foram removidas do frasco de cultivo com Tripsina/EDTA, e quantificadas em câmara de Neubauer. Então as células foram adicionadas em uma mistura de matrigel:meio de cultura (1:2) em gelo, para que a matriz não polimerizasse. A concentração final das células era de 150 células/µL de mistura. Imediatamente depois, 60 µL da mistura contendo células foram adicionadas em cada câmara da lâmina de cultivo e a lâmina foi colocado a 37ºC por uma hora para que o gel polimerizasse. Em seguida 300 µL de meio suplementado com 2% de soro fetal bovino (SBF) foi adicionado em cada câmara. A partir de então o meio foi trocado a cada dois dias, sem suplementação com SFB. Em um dos experimentos a lamina foi levada ao microscópio invertido, com câmara de CO_2 5%, e temperatura 37ºC e submetido a imagens *time-lapse* com espaçamentos dos frames de dez minutos.

Por fim, os diferentes tratamentos, de acordo com indicado nos resultados foram feitos com as células cultivadas em 3D e depois as mesmas foram submetidas a fixação em PFA 4%, lavadas em PBS, e marcadas com faloidina-TRITC e Dapi, para que depois imagens de cinco campos por câmara foram feitas no microscópio confocal Zeiss LSM780 do INCT-INFABiC (UNICAMP, Campinas, SP), para serem analisadas.

Analise de imagens e de dados

As analises de dados e estatísticas foram realizadas no Prims 6 for Mac OS X (© 1994 – 2014 GraphPad Software, Inc. All rights reserved, La Jolla, CA, USA) e as analises e processamento de imagens foram realizadas no ImageJ (Version 2.0.0-rc-31/1.49v, NIH, Bethesda, MD, USA).

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Ata da Sessão Pública de Defesa de TESE para obtenção do Título de DOUTOR em BIOLOGIA CELULAR E ESTRUTURAL, na área de BIOLOGIA CELULAR, a que se submeteu o aluno GUILHERME OLIVEIRA BARBOSA, RA 073180, orientado pelo Prof. Dr. HERNANDES FAUSTINO DE CARVALHO.

Aos trinta e um dias do mês de agosto do ano de dois mil e quinze, às 14:00 horas, na Sala de Defesa de Tese do Prédio da Coordenadoria de Pós-Graduação do Instituto de Biologia da Universidade Estadual de Campinas, reuniu-se a Comissão Examinadora da Defesa em epígrafe indicada pela Comissão de Pós-Graduação do Instituto de Biologia, composta pelos Doutores: **HERNANDES FAUSTINO DE CARVALHO** (Presidente e Orientador), Professor Titular do Departamento de Biologia Estrutural e Funcional do Instituto de Biologia da Universidade Estadual de Campinas, SP, **MARIA APARECIDA DA SILVA PINHAL**, Professora Titular da Faculdade de Medicina Fundação ABC, Santo André, SP, **ENILZA MARIA ESPREAFICO**, Professora Doutora do Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos da Faculdade de Medicina da Universidade de São Paulo, Campus de Ribeirão Preto, SP, **CLAUDIO CHRYSOSTOMO WERNECK**, Professor Doutor do Departamento de Bioquímica e Biologia Tecidual do Instituto de Biologia da Universidade Estadual de Campinas, SP e **MÁRIO HENRIQUE BENGTSON**, Professor Doutor do Departamento de Bioquímica e Biologia Tecidual do Instituto de Biologia da Universidade Estadual de Campinas, SP, para analisar o trabalho do candidato **GUILHERME OLIVEIRA BARBOSA**, apresentado sob o título: "**O eixo heparam-sulfato/Heparanase1 na morfogênese epitelial de próstata**". O Presidente declarou abertos os trabalhos, a seguir o candidato dissertou sobre o seu trabalho e foi arguido pela Comissão Examinadora. Terminada a exposição e a arguição, a Comissão reuniu-se e deliberou pelo seguinte resultado:

[X] **APROVADO**

[ ] **APROVADO CONDICIONALMENTE** (ao atendimento das alterações sugeridas pela Comissão Examinadora especificadas no parecer anexo).

[ ] **REPROVADO** (anexar parecer circunstanciado elaborado pela Comissão Julgadora).

Para fazer jus ao Título de DOUTOR em BIOLOGIA CELULAR E ESTRUTURAL, na área de BIOLOGIA CELULAR, a versão final da TESE, considerada Aprovada ou Aprovada Condicionalmente devidamente conferida pela CPG da Unidade, deverá ser entregue à CPG dentro do prazo de 60 dias, a partir da data da defesa. De acordo com o previsto na Deliberação CONSU-A8/08, Artigo 35, parágrafo 1°, inciso II e parágrafo 2°, o aluno Aprovado Condicionalmente que não atender a esse prazo será considerado Reprovado. Após a entrega do exemplar definitivo, o resultado será homologado pela Comissão Central de Pós-Graduação da UNICAMP, conferindo título de validade nacional aos aprovados.

Nada mais havendo a tratar, o Sr. Presidente declara a sessão encerrada, sendo a ata lavrada por mim, que segue assinada pelos Senhores Membros da Comissão Examinadora, pela Coordenadora da Comissão de Pós-graduação, com ciência do aluno.

Prof. Dr. HERNANDES FAUSTINO DE CARVALHO
Presidente da Comissão Julgadora

Profa. Dra. MARIA APARECIDA DA SILVA PINHAL

Profa. Dra. ENILZA MARIA ESPREAFICO

Prof. Dr. CLAUDIO CHRYSOSTOMO WERNECK

Prof. Dr. MÁRIO HENRIQUE BENGTSON

Profa. Dra. VALÉRIA HELENA ALVES CAGNON QUITETE
Coordenadora da CPPG-Biologia Celular e Estrutural

LILIAM ALVES SENNE PANAGIO
Secretária de Pós-Graduação

Ciência do aluno: GUILHERME OLIVEIRA BARBOSA

Profa. Dra. Sandra Maria Carmello Guerreiro
Coordenadora de Pós-Graduação/IB/UNICAMP
Matrícula 27185-3

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Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto **"Atividade da heparanase e seu papel na modulação da sinalização extracelular no desenvolvimento prostático"** (protocolo nº **2920-1**), sob a responsabilidade de **Prof. Dr. Hernandes Faustino Carvalho / Guilherme Oliveira Barbosa**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **17 de fevereiro de 2014**.

Campinas, 17 de fevereiro de 2014.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

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Profa. Dra. Rachel Meneguello
Presidenta
Comissão Central de Pós-Graduação
Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Tese de Doutorado, intitulada "O eixo heparam-sulfato/Heparanase1 na morfogênese epitelial de próstata", não infringem os dispositivos da Lei nº 9.610/98, nem o direito autoral de qualquer editora.

Campinas, .

Autor(a): Guilherme Oliveira Barbosa
RG nº 2.029.657 SSP-ES

Orientador(a): Hernandes F. Carvalho
RG nº 32.190.095-9 SSP-SP