

UNIVERSIDADE ESTADUAL PAULISTA

“JÚLIO DE MESQUITA FILHO”

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

**Esteroidogênese na prole de ratos submetidos à restrição
proteica materna: avaliação e estudos funcionais de
microRNAs/mRNAs responsivos à estrógeno na próstata**

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Luiz Marcos Frediani Portela*Discente***Luís Antônio Justulin Junior***Orientador***Erick José Ramo da Silva***Coorientador***Sérgio Alexandre Alcantara dos Santos***Colaborador*

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Biotecnologia, Campus de Botucatu, UNESP,
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Prof. Dr. Luís Antônio Justulin Junior

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Lista de abreviações

AR – Receptor de Andrógeno

CaP- Câncer de Próstata

CTR- Grupo Controle

CYP- Enzimas do complexo Citocromo p450

DHEA- Desidroepiandrosterona

DHT- Di-hidrotestosterona

DOHaD- Origem Desenvolvimentista da Saúde e da Doença (do inglês, Development Origins Health and Disease)

ER α – Receptor de estrógeno alfa

ER β – Receptor de estrógeno beta

FOAD – Fetal Origin of Adults Disease

GPER1 – Receptor de Estrógeno acoplado a proteína G 1

HSD- Hidroxiesteroide desidrogenase

INCA- Instituto Nacional do Câncer

GLLP – Grupo Restrição proteica materna gestacional e lactacional (do inglês, gestational and lactational low protein)

ncRNA- RNAs não codificantes

OMS – Organização Mundial da Saúde

ONU- Organização das Nações Unidas

PF- Programação Fetal

PV- Próstata Ventral

RPM- Restrição Proteica Materna

UNICEF- Fundo das Nação Unidas para a Infância

SUG - epitélio do seio urogenital

MEC - matriz extracelular

DG - Dia gestacional

CPRC - Câncer de Próstata Resistente a Castração

RAR - receptores retinoides

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RESUMO

A restrição proteica materna (RPM) é um fator determinante na alteração da saúde da prole ao longo da vida, colocando-a dentro da abordagem da Origem Desenvolvimentista da Saúde e da Doença (DOHaD). Está associada a RPM alterações nos níveis séricos de hormônios esteroides, perda do desenvolvimento normal da próstata e torna-se um fator determinante para o aumento da incidência de câncer de próstata (CaP) com o envelhecimento. Descrevemos aqui que os animais que foram submetidos a RPM no dia pós-natal (DPN) 21 apresentam baixo peso ao nascer, diminuição dos níveis séricos de progesterona, dehidroepiandrosterona, aumento do colesterol total, testosterona e estrogênio. Associamos o aumento desses hormônios, principalmente estrogênio, com o aumento da expressão de *Cyp19a1* (aromatase) na pele, no fígado e na glândula adrenal. Como resultado desse aumento nos níveis de estrogênio, observamos uma perda do desenvolvimento normal do lobo prostático ventral (PV) levando a glândulas menores e alteração na expressão genica de receptores hormonais. Estudos de sequenciamento de miRNA mostraram o miR206-3p up-regulado na PV desses animais, através de análises *in silico* identificamos que os alvos preditos para miR206-3p estão potencialmente contribuindo para a carcinogênese prostática, esses alvos levam a pior prognóstico de sobrevida em pacientes humanos com PCa. Ensaio que mimetizam a expressão de miR206-3p em células prostáticas humanas benignas da linhagem PNT2, mostraram uma redução na viabilidade celular como resultado do aumento do miR206-3p. Com isso descrevemos como a RPM impacta a esteroidogênese sistêmica da prole, e a possível influência do miR206 na resposta prostática a essa condição adversa

Palavras-Chave: DOHaD; Restrição Proteica Materna; Próstata Ventral; esteroidogênese; miRNA

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Capítulo 1. Revisão de Literatura

1. Enunciado da problemática

Apesar do direito de nenhum indivíduo padecer de fome e desnutrição, esta é a segunda causa que mais mata indivíduos com até cinco anos de idade em países subdesenvolvidos e/ou em desenvolvimento (BHUTTA et al., 2017). Dados do Fundo das Nações Unidas para a Infância (UNICEF) e da Organização Mundial de Saúde (OMS) estimam que cerca de 821 milhões de pessoas no mundo sejam subnutridas, além de estimarem que entre 1990 e 2015 cerca de 263 milhões de indivíduos com até cinco anos vieram a óbito no mundo devido à desnutrição (MATHERS et al., 2015). A Organização das Nações Unidas (ONU), ao estabelecer os “*Objetivos do Desenvolvimento Sustentável*”, destacou em sua meta número 2, a “*Fome Zero e agricultura sustentável*”, para mostrar ao mundo a importância da redução da desnutrição (ONU, 2015).

São reconhecidos vários tipos de desnutrição, dentre eles a desnutrição proteica, que é caracterizada pela diminuição na oferta de aminoácidos, que leva ao desequilíbrio na homeostasia sistêmica do indivíduo (BORCK et al., 2017). O tipo mais prevalente de desnutrição é aquele que compromete o crescimento somático do indivíduo, dando um fenótipo de baixo peso, baixa estatura e anormalidades fisiológicas (BLACK et al., 2013). Outra problemática associada à desnutrição é a maior susceptibilidade a resposta a doenças infecciosas e agudas (ZMORA et al., 2017). Como por exemplo, durante a epidemia de Zika vírus no Brasil e no mundo entre os anos de 2016 e 2017, muito se discutiu sobre os cofatores que contribuíam para as diferentes formas da Síndrome Congênita do Zika (SCZ) (DE ARAÚJO et al., 2018). Barbeito-Andres e seus colaboradores (2020), em um estudo experimental com roedores submetidos a desnutrição materna e expostas ao Zika vírus apresentavam alterações na estrutura placentária, menor crescimento da prole, diminuição da neurogênese e o fenótipo de microcefalia (BARBEITO-ANDRÉS et al., 2020). Assim, a desnutrição proteica se torna ainda mais grave ao afetar mulheres em idade reprodutiva, recém-nascidos e crianças de até cinco anos de idade.

Nas últimas décadas inúmeros trabalhos tentaram compreender a correlação da maior incidência de doenças crônicas na vida adulta com insultos sofridos no período intrauterino e/ou primeira infância. Tais insultos podem ser o consumo de drogas e tabaco, alimentação inadequada, poluição ambiental, e doenças metabólicas que podem

afetar de maneira irreversível o desenvolvimento do embrião/feto (MERICQ et al., 2017). Em 1977, Forsdahl foi um dos primeiros cientistas a publicar trabalho correlacionando a incidência de doenças ateroscleróticas na vida adulta com a pobreza na infância e adolescência (FORSDAHL, 1977). No entanto, anos mais tarde, durante a década de 1980, o médico epidemiologista inglês David Barker e seus colaboradores, demonstraram a correlação positiva entre má nutrição infantil, aumento da incidência de mortalidade neonatal e de aumento de mortes por doenças cardíacas na idade adulta, em regiões mais pobres da Inglaterra e do País de Gales (BARKER; OSMOND, 1986), ainda na década de 1980 Barker e colaboradores mostraram que recém-nascidos entre 1911 e 1930 na região de Hertfordshire-Inglaterra e que apresentavam um baixo peso corpóreo para a idade, tinham até 2,7 mais chances de morrer em decorrência de doenças cardíacas quando comparados à recém-nascidos com peso considerado normal para a idade (BARKER et al., 1989). No início da década de 1990 em outro estudo, David Barker e seus colaboradores, analisando dados de peso corpóreo ao nascimento, peso da placenta, peso corpóreo na vida adulta e pressão arterial conseguiram demonstrar que o baixo peso corpóreo ao nascimento associado à maior peso placentário materno estavam diretamente associados aos quadros de hipertensão na vida adulta, dando origem à **“Origem fetal das doenças do adulto”** (*“Fetal Origin of Adult Diseases”*) **(FOAD)** (BARKER et al., 1990). Posteriormente, em 1991, Alan Lucas cunhou o termo **Programação Fetal (PF)** (LUCAS, 1991). Hales e Barker em 1992, propuseram a ideia do “fenótipo econômico”, que a PF por restrição alimentar nas fases iniciais do desenvolvimento adapta o metabolismo a essa condição pobre em nutrientes, e se na vida adulta tivesse contato com uma alimentação normal estaria mais propenso a desenvolver obesidade e diabetes mellitus do tipo 2 (HALES; BARKER, 1992). Essa teoria e estudos epidemiológicos, inicialmente controversos, despertaram grande interesse médico-científico e anos mais tarde culminou na criação de uma sociedade internacional, denominada **Origem desenvolvimentista da Saúde e da Doença** (*“Developmental Origins Of Health and Disease”*) (DOHaD) (SUZUKI, 2018).

Entre os anos de 1944 e 1945, próximo ao final da 2ª Guerra Mundial a Alemanha visando a rendição da Holanda, impediu a chegada de alimentos a esse país, ocasionando um período de severa escassez alimentar, onde o consumo alimentar dessa população foi limitado a 400-800 calorias/dia, inclusive para gestantes (SCHULZ, 2010). Esse episódio ficou conhecido como *“Dutch Hunger Winter”* (Inverno da Fome

Holandês). Embora seja um evento trágico os dados dessas populações nos permitiram compreender como a má alimentação materna impacta sobre a saúde humana, corroborando com a teoria de Barker. Ravelli et al. (1976) demonstraram o impacto da restrição alimentar sobre os filhos das mulheres gestantes durante o Inverno da Fome Holandesa. Na vida adulta, esses indivíduos cujas mães sofreram restrição alimentar, apresentaram padrões diferenciados de composição corporal dependendo da fase da gestação em que haviam sido expostos à desnutrição materna, sendo o trimestre inicial responsável por maior índice de obesidade quando comparadas ao último trimestre (RAVELLI; STEIN; SUSSER, 1976). Com isso, os autores demonstraram que, além do impacto negativo da desnutrição materna sobre a prole, seus efeitos dependem do período gestacional em que o insulto ocorre.

Ao longo do tempo, outros trabalhos começaram a ser publicados descrevendo maiores informações sobre essas vítimas do Inverno da Fome Holandesa. Hoek e Susser, estabeleceram que a desnutrição materna estava associada à anomalias congênitas no sistema nervoso central, aumento dos casos de esquizofrenia, bem como distúrbios de personalidades (HOEK; BROWN; SUSSER, 1998), e potencialmente se tornam mais propensos a desenvolverem vícios durante a vida adulta (FRANZEK et al., 2008). Outros estudos sugerem que esses indivíduos apresentavam uma diminuição na secreção de insulina, assim ocasionando menor tolerância a glicose e o desenvolvimento de diabetes mellitus tipo 2 (DE ROOIJ et al., 2006); foi estabelecido que mulheres descendentes da fome holandesa não apresentavam alteração no sucesso reprodutivo embora um quarto delas entravam na menopausa precocemente (YARDE et al., 2013).

1.1 Programação Fetal por restrição proteica materna

A restrição proteica materna (RPM) em ratas gestantes e/ou lactantes é um dos modelos mais conhecidos e utilizados em estudos de PF (JAHAN-MIHAN et al., 2015; LANGLEY-EVANS; MCMULLEN, 2010; LANGLEY-EVANS; PHILLIPS; JACKSON, 1994). A literatura reporta que a RPM durante a gestação e/ou lactação é responsável pelo baixo peso da prole ao nascimento (COLOMBELLI et al., 2017; SANTOS et al., 2018). Além disso, esses indivíduos com baixo peso ao nascer e que desenvolvem crescimento pós-natal acelerado (*catch-up growth*) apresentam maior propensão a desenvolver resistência à insulina e diabetes tipo 2 na idade adulta (BERENDS et al., 2013, 2018; KESAVAN; DEVASKAR, 2019).

Estudos experimentais mostram que a RPM se torna fator determinante para o desenvolvimento de síndromes metabólicas na prole ao longo da vida (JAHANMIHAN et al., 2015), altera a resposta do indivíduo frente às condições adversas (MARTINS et al., 2018) e está associada a alterações morfofuncionais em diferentes órgãos, evidenciando que a RPM altera sistemicamente o indivíduo. Sendo relatado modulação do metabolismo de glicose (ZHENG et al., 2017), desbalanço nos níveis séricos de leptina (QASEM et al., 2016; ZAMBRANO et al., 2006), alteração nas concentrações séricas de Insulina, IGF1 e IGF2 (BERENDS et al., 2018; COLOMBELLI et al., 2017; SANTOS et al., 2018), de moléculas inflamatórias como IL1b (TARRY-ADKINS et al., 2018) e de hormônios esteroides (RINALDI et al., 2013; SANTOS et al., 2018).

Além dessas alterações metabólicas, há também alterações órgão-específicas, como: diminuição dos depósitos de gordura, diminuição da proliferação celular no fígado, além de alteração na expressão gênica e em vias importantes para a sobrevivência dos hepatócitos (CAMPISANO et al., 2017; MARTIN et al., 2018; RAMADAN; ALSHIRAIHI; AL-KARIM, 2013); menor quantidade do número de células β , menor taxa de proliferação celular, perda da sensibilidade de resposta no pâncreas às alterações metabólicas e diminuição na secreção de insulina (DUMORTIER et al., 2014; MORIMOTO et al., 2012; SU et al., 2016); alteração na arquitetura, bioquímica e nas proporções de neurônios no cérebro (GOULD et al., 2018), atrofia dendríticas em neurônios hipotalâmicos (LOPES et al., 2013), bem como alteração de neurônios e vias hipotalâmicas associadas ao controle da saciedade (COUPE et al., 2009; MARTIN-GRONERT et al., 2016); diminuição do número de néfrons ao longo da vida (RIZZI et al., 2017) e alteração de vias moleculares que regulam a transição epitélio-mesênquima no rim (SENE et al., 2018); menor número de alvéolos pulmonares, redução da vascularização pulmonar e alteração na produção de surfactante (KHAZAEI et al., 2019; LIU et al., 2014); quadros de hipertensão, cardiopatias, mudanças na morfologia e em vias moleculares no coração (ASSALIN; GONTIJO; BOER, 2019); alteração na estrutura morfológica e da microbiota intestinal, bem como diminuição da absorção de ferro e nutrientes no intestino (JIN et al., 2019; MA et al., 2017; WANG et al., 2020); alteração no padrão da musculatura estriada esquelética (CABEÇO et al., 2012) e diminuição da massa óssea (QASEM et al., 2016).

Nas últimas décadas foram descritos alguns dos impactos que a RPM tem sobre o sistema genital, sendo observado redução da distância ano-genital na prole masculina (SANTOS et al., 2018) e aumento nas fêmeas (GUZMÁN et al., 2006), diminuição da taxa de fertilidade e alteração da instalação da puberdade (ZAMBRANO et al., 2014). No sistema genital da prole feminina foram observadas diminuição do número da reserva de folículos ovarianos e alteração no padrão de produção de hormônios esteroides intraovariano (GUZMÁN et al., 2014); além do atraso no desenvolvimento mamário (BAUTISTA et al., 2013) e favorecimento no desenvolvimento de câncer de mama após exposição ao desregulador endócrino bisfenol A (BPA) (VARUZZA et al., 2019). Além disso, na prole masculina foram descritos diminuição do peso testicular, alteração na organização celular dos túbulos seminíferos, redução da expressão do receptor de andrógeno nos testículos e comprometimento da produção espermática (RODRÍGUEZ-GONZÁLEZ et al., 2012), seguido de aumento de estresse oxidativo intratesticular e espermático (RODRÍGUEZ-GONZÁLEZ et al., 2014); alteração morfológicas e morfométrica do epidídimo e redução da vasculogênese do epidídimo nas fases iniciais do desenvolvimento epididimal e na idade reprodutiva (CAVARIANI et al., 2019; DE MELLO SANTOS et al., 2019). Esses dados, em conjunto demonstram que a RPM afeta negativamente o status metabólico, a morfogênese e a homeostase de importantes órgãos e tecidos ao longo da vida do indivíduo, podendo comprometer sua capacidade e sucesso reprodutivo.

1.2 Desenvolvimento prostático e Restrição proteica materna

Dentre os órgãos reprodutivos que são essenciais para o sucesso reprodutivo temos a próstata que é uma glândula exócrina acessória ao sistema genital masculino, cujo desenvolvimento e homeostase estão sobre controle de andrógenos, ela é dividida em dois compartimentos, o estroma e epitélio, que sinalizam de forma parácrina desencadeando inúmeras respostas moleculares (CUNHA et al., 1987, 2018; MARKER et al., 2003). Ela é responsável por secretar um complexo proteolítico composto por fosfatase ácida, ácido cítrico, fibrinolizina, enzimas específicas, imunoglobulinas que ajudam a compor o fluido seminal (VERZE; CAI; LORENZETTI, 2016). Essa secreção é importante para o sucesso da reprodução, auxilia na motilidade espermática, uma vez que ele liquefaz o ejaculado, além de garantir nutrientes e enzimas que irão auxiliar a alcalinizar o canal vaginal, permitindo que o espermatozoide caminhe até o ovócito (MARKER et al., 2003; VERZE; CAI; LORENZETTI, 2016). Mas a principal proteína

secretada pela próstata é o antígeno prostático específico (PSA), uma molécula pertencente à família das calicreínas, uma protease que contribui para impedir a coagulação do fluído seminal (WANG et al., 2017a); além de sua importância fisiológica o PSA é usado como valor prognóstico e diagnóstico na clínica do Câncer de Próstata (CaP) (MORADI et al., 2019; NORDSTRÖM et al., 2018).

Anatomicamente, a próstata humana está localizada no compartimento subperitoneal entre o diafragma pélvico e a cavidade peritoneal. Ela tem uma base ligada ao colo da bexiga, a face anterior é separada da sínfise púbica pela gordura retroperitoneal no espaço retropúbico, a face posterior relacionada com a ampola do reto e as faces inferolaterais relacionadas com o músculo levantador do ânus. Ela tem o formato de “noz”, que em condições normais, pesa cerca de 20-30 gramas e circunda a parte prostática da uretra (HENRY et al., 2018; ITTMANN, 2018). Embora seja uma estrutura única a próstata é subdividida em três conjuntos de estruturas túbulo alveolares formando zonas, sendo elas: Zona central, região mais próxima a uretra e que corresponde a 5% da fração glandular; Zona de transição região mediana, cuja região corresponde a 25% da fração glandular, essa região se torna extremamente importante uma vez que ela é acometida nos quadros de Hiperplasia Prostática Benigna (HPB); e a Zona Periférica, que corresponde a 70% de sua extensão e essa região que é acometida no CaP. Externamente, a próstata é envolta por uma capsula fibroelastica (TOIVANEN; SHEN, 2017; YACOUN; OTO, 2018). Diferente da próstata humana, os roedores apresentam uma próstata dividida em quatro pares de lobos disposto ao redor da uretra, o lobos prostáticos em roedores são: ventral, dorsal, lateral e anterior (MARKER et al., 2003). Cada lobo prostático possui suas particularidades de organização histológica, padrão de expressão gênica e responsividade aos hormônios (CUNHA et al., 2018; MARKER et al., 2003; PRINS; PUTZ, 2008) . Na Figura 1 estão representadas principais diferenças anatômicas entre a próstata de humano e roedores.

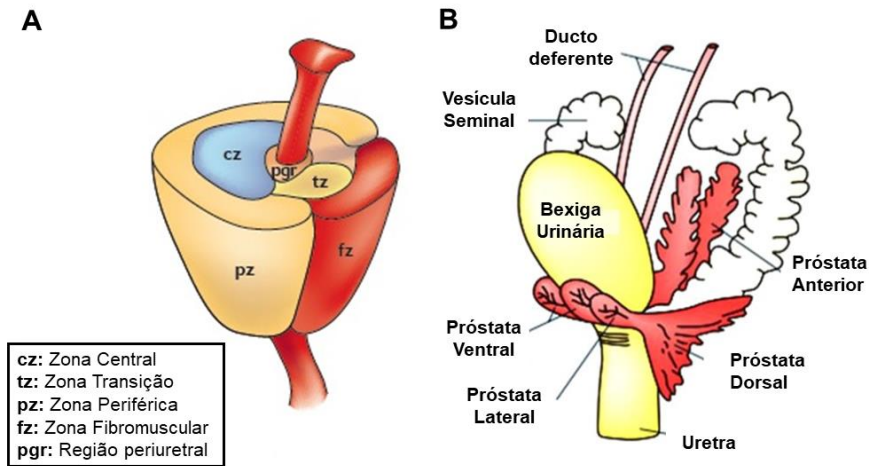


Figura 1. Imagem representativa da anatomia prostática. A: Representação da anatomia da Próstata humana, adaptado de VERZE; CAI; LORENZETTI (2016). B: Representação da anatomia da Próstata de roedores, imagem adaptada de CUNHA (1985).

Assim como na próstata humana a próstata de roedores possui estruturas túbulo-alveolares que são revestidas por células epiteliais simples. A população de células encontradas na próstata podem ser: as células secretoras/luminais, a maior população de células prostáticas e que são as células responsáveis por secretar as proteínas que irão compor o fluido seminal; as basais, presentes em menor número, que possuem função proliferativa, mantendo as populações celulares da glândula; as intermediárias que apresentam características intermediárias entre as células secretoras e basais; as neuroendócrinas, que são àquelas que atuam orquestrando o desenvolvimento e manutenção da próstata, além de secretar inúmeros fatores de crescimento; ambas células ancoradas em uma membrana basal composta por colágeno do tipo IV e laminina (TOIVANEN; SHEN, 2017). Em seu estroma há presença de células musculares lisas, fibroblastos, neurônios, vasos, além de mastócitos e macrófagos e toda uma rede complexa de elementos de matriz extracelular (MEC), rica em colágeno, fibras reticulares, fibras elásticas, proteoglicanos e glicoproteínas (Figura 2) (MCNEAL, 1988; TOIVANEN; SHEN, 2017; YACOUN; OTO, 2018).

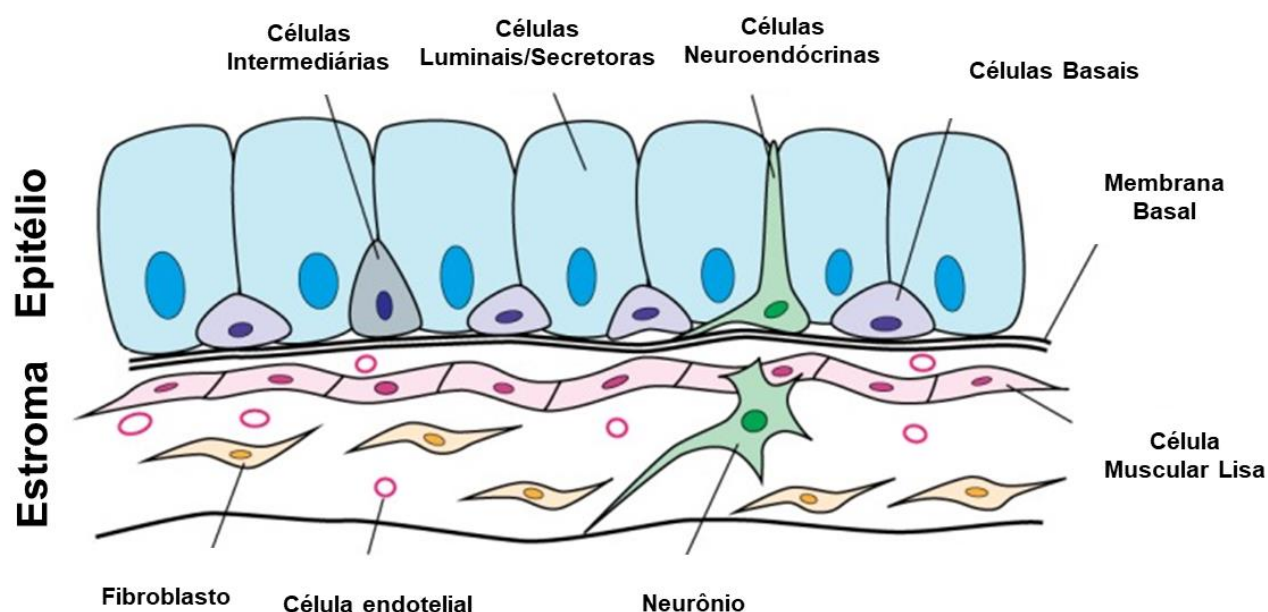
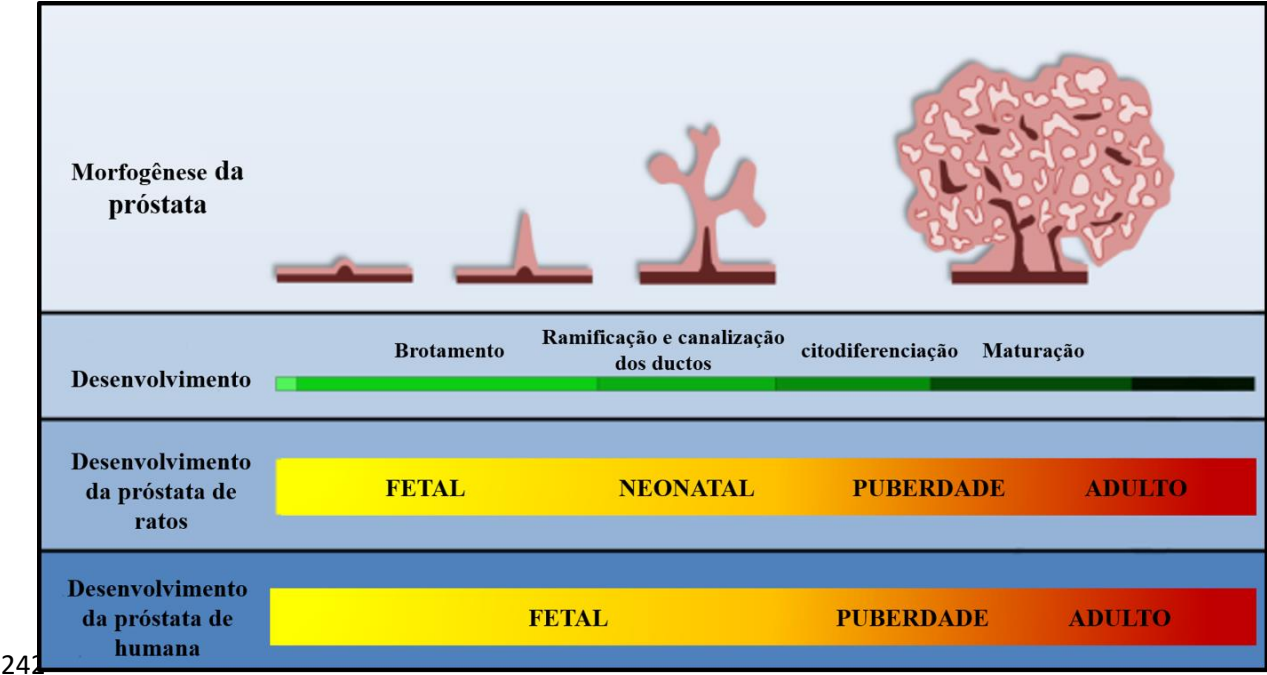


Figura 2. Imagem representativa da organização tecidual da próstata, sendo representado os componentes do epitélio e do estroma, imagem adaptada de TOIVANEN e SHEN (2017).

Em humanos o desenvolvimento da próstata se inicia por volta da 9,5ª semana, em roedores ela tem início no dia gestacional (DG) 17º em camundongos e 18º em ratos (CUNHA et al., 1987, 2018). Em ambas as espécies o desenvolvimento da próstata se dá por um processo altamente conservado, chamado de “Morfogênese de Ramificação”, onde pequenos brotos se projetam a partir do epitélio do seio urogenital (SUG) e que adentra no mesênquima que o circunda (FRANCIS; SWAIN, 2018; THOMSON, 2008). Enquanto em humanos o desenvolvimento prostático acontece a maior parte no período intrauterino, em roedores a maior parte acontece na vida pós natal, nos primeiros 15 dias de vida (SCARANO et al., 2018). Mesmo com essa particularidade, em ambas as espécies o processo de desenvolvimento prostático encerra sua maturação com a chegada da puberdade (MARKER et al., 2003).

Em roedores, o início do desenvolvimento prostático se dá entre o DG 13-14 quando as células de Leydig dos testículos fetais começam a produzir testosterona, que leva a um pico da concentração hormonal no DG 15 (CUNHA et al., 2018; TIMMS, 2008). Em resposta a esse aumento nos níveis hormonais, uma região específica do epitélio do SUG no DG15,5 sofre alteração no seu padrão de sinalização intracelular em resposta a ativação do Receptor de Andrógeno (AR), essas células irão dar origem a próstata, esse processo é denominado determinação, as vias ativadas nesse momento são de *p63*, *Wnt* e *Sonic hedgehog (Shh)* (PRINS; PUTZ, 2008; SCARANO et al., 2018;

230 TOIVANEN; SHEN, 2017). Para que ocorra a formação dos primeiros brotos
 231 prostáticos, através de sinalização parácrina entre epitélio e mesênquima, é ativada as
 232 vias de Fatores de Crescimento de Fibroblasto (*Fgf*), *Wnt* e *Sox9* (FRANCIS; SWAIN,
 233 2018; PRINS; PUTZ, 2008). O alongamento, ramificação e canalização dos ductos
 234 prostáticos acontece em roedores no período neonatal, enquanto em humanos ocorre *in*
 235 útero, para essa etapa é necessária a ativação de vias *Hox*, *Nkx3.1*, *Fgf*, *Wnt*, *Bmp* e
 236 *Notch*, que irão orquestrar a proliferação e a ramificação da próstata (ABATE-SHEN;
 237 SHEN; GELMANN, 2008; PRINS; PUTZ, 2008; SCARANO et al., 2018; ZHU et al.,
 238 2007). A citodiferenciação da próstata acontece na puberdade e nela as células passam a
 239 expressar *Ar*, *Pten*, *FoxA1*, *Hox*, *Nkx3.1* e *Fgf*, esse processo é essencial para que a
 240 próstata enfim consiga chegar a sua maturidade (CUNHA et al., 2018; FRANCIS;
 241 SWAIN, 2018; MARKER et al., 2003; PRINS; PUTZ, 2008) (Figura 3).



243 **Figura 3.** Esquema representativo do desenvolvimento da próstata em roedores e humanos, adaptado de
 244 SCARANO et al., (2018)

245 Além de ser importante para fertilidade a próstata nos últimos anos vem
 246 despertado grande interesse médico-científico pela alta incidência de patologias, dentre
 247 elas destaca-se a HPB e o CaP (BANERJEE et al., 2018). Segundo dados publicados
 248 pela “*Global Cancer Statistics*” (Globocan) 2018, o CaP é o segundo câncer que mais
 249 acomete homens no mundo ficando atrás apenas do câncer de pele não melanoma e a
 250 quinta principal causa de mortes no mundo, sendo diagnosticado no mundo 1.276.106
 251 novos casos apenas em 2018, sendo mais comum o diagnóstico em países

desenvolvidos, atingindo homens com idades acima de 65 anos e mais comum em homens afro-americanos que homens brancos (BRAY et al., 2018). Dados do Instituto Nacional do Câncer (INCA), mostra que a realidade do CaP não é diferente no Brasil, a estimativa para o ano de 2020 é de 65.840 novos casos (INCA, 2020).

Nos últimos anos, nosso grupo de pesquisa vem se dedicando a entender como a RPM afeta o desenvolvimento da próstata e seus efeitos até o envelhecimento da prole. Colombelli et al., (2017) observaram que a próstata ventral (PV) dos animais submetidos à RPM durante a gestação e/ou lactação apresentaram menor tamanho dos ácinos prostáticos, diminuição da angiogênese da glândula e queda nos níveis séricos de testosterona no dia pós-natal (DPN) 10 e 21. Santos et al., (2018; 2020) investigaram o período final do desenvolvimento prostático e o envelhecimento, com animais nos DPN 21 e 540, notando atraso no desenvolvimento da PV no DPN 21 e maior incidência de lesões prostáticas no envelhecimento, tais como displasia epitelial, neoplasia intraepitelial (PIN) e carcinoma *in situ* nos animais submetidos a RPM, estes dados foram correlacionados à estrogenização desses animais, pois foram observados altos níveis séricos de estrógenos nas mães e prole no DPN 21 e aumento na proporção estrógeno/testosterona nos animais no DPN 540, outro fator importante foi a alteração no perfil proteômico da glândula prostática desses animais, apresentando o enriquecimento de vias moleculares associadas ao perfil de perturbação estrogênica (SANTOS et al., 2020). Portela e seus colaboradores (2020), mostraram que esses animais no DPN 540 apresentavam um aumento do estresse oxidativo intraprostático e desregulação na expressão de importantes biomarcadores conhecidos do CaP (PORTELA et al., 2020).

1.3 Esteroidogênese

O estrógeno é um hormônio esteroide, de natureza lipídica, cuja síntese se dá através de uma série de reações em cascata a partir de uma molécula de colesterol, o processo de formação dos hormônios esteroides recebe o nome de esteroidogênese (Figura 4). Num primeiro momento há conversão do colesterol em pregnenolona mediado pela enzima CYP11a1 dentro das mitocôndrias. No citoplasma a pregnenolona pode seguir dois caminhos, em um deles é convertida em progesterona pela enzima 3 β HDS. A progesterona é convertida pela p450c17 em 17OH-Progesterona e pela 17,20 liase é convertida em androstenediona.; no outro caminho a pregnenolona é convertida

em 17OH-Pregnenolona pela p450c17, que depois pela 17,20 liase é convertida em Desidroepiandrosterona (DHEA), o DHEA é convertido pela 3 β HDS androstenediona, sendo essa convertida pelo 17 β HDS em testosterona. A testosterona pode ser metabolizada, gerando duas moléculas: a DHT via enzima 5 α redutase; ou o estrógeno via aromatase (ACKERMANN et al., 2019; MILLER, 2017). Os processos de esteroidogênese acontecem tanto em órgãos reprodutivos como testículo e a próstata, bem como em órgãos periféricos, em órgãos centrais do sistema endócrino como adrenal, fígado e pele.

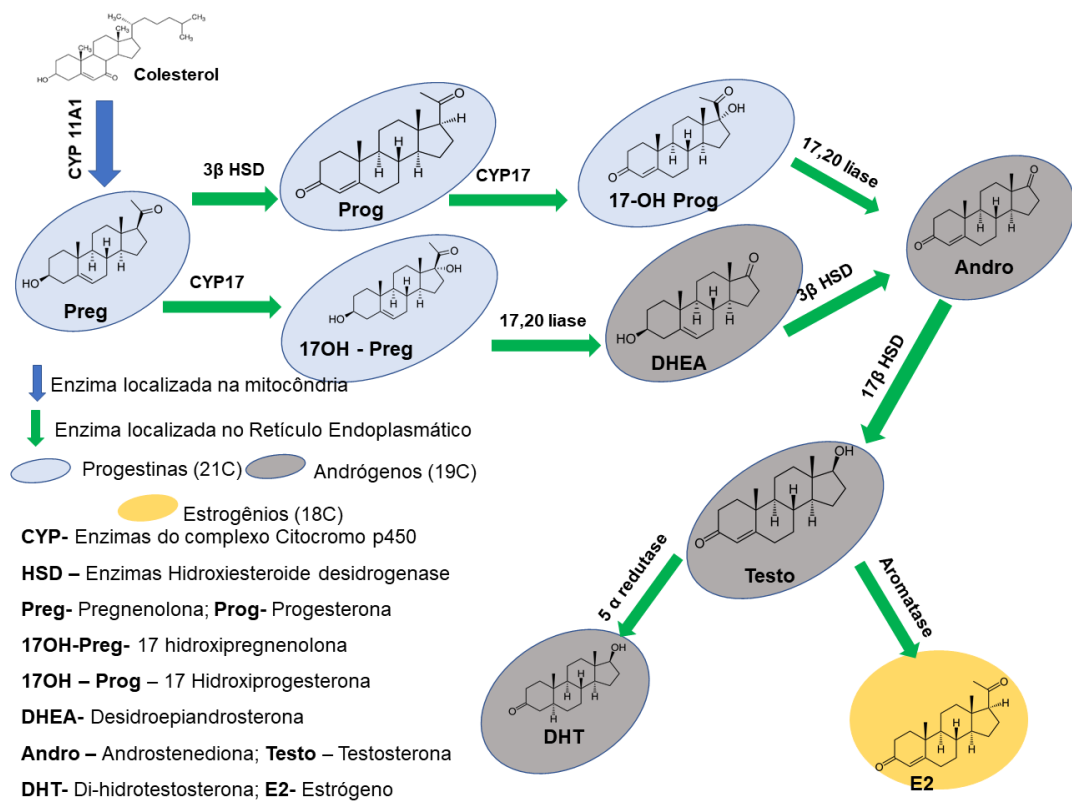


Figura 4. Imagem representativa das etapas bioquímicas da esteroidogênese.

Nos testículos, em específico nas células de Leydig, àquelas localizadas no interstício entre os túbulos seminíferos, elas captam moléculas de colesterol da corrente sanguínea e realizam o processo de conversão à testosterona (WANG et al., 2017b). Em ratos tratados com corticosterona e 2,3,7,8-tetracloro dibenzo-p-dioxina (TCDD), apresentaram baixos níveis intratesticulares de testosterona e uma drástica diminuição na expressão de enzimas que atuam na esteroidogênese (DHANABALAN; MATHUR; LATHA, 2015); o antibiótico triclosan também é outro composto que leva a redução dos níveis de testosterona intratesticular, diminuição da expressão gênica e proteica de enzimas 3 β Hsd e 5 α -redutase (HA et al., 2018); também foi demonstrado que animais

diabéticos apresentam diminuição nos níveis séricos de testosterona, hormônio luteinizante (LH), diminuição da expressão intratesticular das enzimas 17β Hsd, Cyp11a1, além da redução da viabilidade espermática (GHOLIZADEH et al., 2019).

Nas glândulas adrenais a esteroidogênese ocorre no seu córtex, ocorrendo processos específicos em cada uma de suas zonas: na zona glomerulosa ocorre a produção de mineralocorticoides, como a aldosterona; na zona fasciculada ocorre a síntese de corticoides, como cortisol e corticosterona; e na zona reticulada a produção de andrógenos, como DHEA e testosterona (VINSON, 2016). Melau e seus colaboradores (2019) caracterizaram a expressão de enzimas esteroidogênicas, os níveis de hormônios intra-adrenais e a localização tecidual de marcadores esteroidogênicos durante o primeiro e o segundo trimestre de gestação de humanos, mostrando que há um perfil distinto em ambas as fases do desenvolvimento (MELAU et al., 2019). Camundongos *knockouts* para proteína translocadora de colesterol para a mitocôndria, demonstraram não alterar os níveis hormonais quando jovens, mesmo com sutis alterações no fluxo da esteroidogênese, no entanto passam a apresentar anormalidades na esteroidogênese ao envelhecerem (BARRON et al., 2018). Estudos demonstraram que a RPM leva a uma diminuição no peso das glândulas adrenais e aumento das concentrações séricas de corticosterona em animais no DPN 91 e DPN 180 (DE OLIVEIRA et al., 2016; FAGUNDES et al., 2007).

Devido a sua capacidade de acumular gotículas de lipídeos em seu interior, o fígado é outra glândula que tem sua homeostase associada aos níveis séricos de hormônios esteroides (JHA et al., 2018), além de apresentar a expressão de inúmeras enzimas da família CYP (CHIKADA et al., 2018). Em condição normal, foi demonstrado que o único hormônio esteroide detectado no fígado é a pregnenolona (MAEDA et al., 2012). Estudos experimentais, mostraram que lesões hepática causadas pela administração de drogas sejam responsáveis por reduzir os níveis séricos de testosterona (ASADI et al., 2017). Murakami e seus colaboradores (2018) demonstraram em pacientes humanos correlação positiva entre o aumento da imunorreação da aromatase e 17β -HSD com fígados lesionados pelo vírus da Hepatite B, sugerindo possível papel da resposta estrogênica nesses processos infecciosos (MURAKAMI et al., 2018). Também foi descrito que a inibição da 5α redutase, seja ela em animais *knockout* ou por meio do inibidor finasterida, estava associado ao aumento

da resistência à insulina e no surgimento de lesões hepáticas (LIVINGSTONE et al., 2015).

A pele também é um importante órgão extra-gonadal, capaz de sintetizar, metabolizar e armazenar hormônios esteroides e glicocorticoides, tendo essa desregulação associada a importantes condições patológicas, como acne e dermatite, muito desse papel se deve pela pele acumular lipídeos na região de hipoderme (NIKOLAKIS et al., 2016). Inoue e seus colaboradores (2012) descreveram a correlação entre os níveis de estrógenos circulantes e níveis de expressão da enzima aromatase na pele humana, além de apresentarem fibras elásticas mais volumosas (INOUE et al., 2012). O cérebro é outro importante órgão cuja sua homeostasia se mostra sensível a variação dos níveis hormonais (FOKIDIS et al., 2015). Durante muito tempo acreditou-se que o cérebro era somente um órgão receptor desses hormônios, até que estudos demonstraram que o cérebro expressava aromatase e que esta tinha a capacidade de converter testosterona a estrógeno (NAFTOLIN; RYAN; DAVIES, 1975), anos mais tarde foi descrita também a presença da 5 α -redutase ativa, na conversão de testosterona a DHT (CELOTTI et al., 1991). Além de ser sintetizado no cérebro esses hormônios, se ligam a receptores regulando funções cerebrais importantes, como mielinização, cognição, lesões celulares e no envelhecimento (ROSSETTI et al., 2016). Estudos com roedores neonatos expostos a BPA apresentavam menor taxa de mielinização no cérebro o que poderia implicar em déficits cognitivos (TIWARI et al., 2015).

Além dos órgãos citados acima, a próstata também desempenha funções associadas a esteroidogênese. Responsiva a andrógenos, a próstata, é responsável por internalizar as moléculas de testosterona e metabolizá-la a di-hidrotestosterona ou estrógeno (CUNHA et al., 1987). Pinski e seus colaboradores demonstram que linhagens de células tumorais de próstata expostas ao LH, eram capaz de produzir testosterona à partir de moléculas de colesterol, aumentando a ativação do receptor de LH e das enzimas CYP11A1, StAR e 3 β HSB (PINSKI et al., 2011). Foi demonstrado também que no Câncer de Próstata Resistente a Castração (CPRC), a diminuição da proliferação celular se mostrou mais efetiva quando foi combinado tratamento com drogas anti-androgênicas em inibição da enzimas CYP (MOLL et al., 2019). Células tumorais tratadas com andrógenos produzidos pela glândula adrenal foram capazes de metabolizar esses andrógenos em testosterona e se associou esse mecanismo à enzimas CYP (KUMAGAI et al., 2013; WU et al., 2019).

1.4 Estrógeno e Próstata

Há cerca de 25 anos, Wilian Gardner em seu trabalho intitulado “*The prenatal origins of prostate cancer*” propôs que a origem do desenvolvimento do CaP estava associada a fatores ocorridos durante o desenvolvimento embrionário e que provavelmente estaria associada a aumento dos níveis sérico de estrógeno (GARDNER, 1995). Outros dados epidemiológicos do CaP também evidenciam que elevados níveis estrogênicos *in útero* estavam associados ao fato de homens afro-americanos apresentarem maior incidência de CaP quando comparados a caucasianos (HENDERSON et al., 1988; POWELL; MEYSKENS, 2001). Em condições normais, o aumento dos níveis de estrógeno intraprostático acontece em dois momentos, no desenvolvimento intrauterino e no envelhecimento (PRINS; KORACH, 2008). O primeiro aumento nos níveis de estrógeno, em humanos, ocorre no terceiro trimestre gestacional, junto a uma queda nos níveis de testosterona, como consequência ao desenvolvimento de metaplasia escamosa no epitélio prostático que regride imediatamente após o nascimento, quando os níveis de estrogênio têm uma queda abrupta (PRINS; KORACH, 2008; WERNERT et al., 1990; ZONDEK et al., 1986). No envelhecimento, o aumento das concentrações séricas de estrógeno está relacionado a maior incidência de lesões prostáticas (KING; NICHOLSON; ASSINDER, 2006; SALINAS et al., 2014; SANTOS et al., 2019).

Prins e seus colaboradores (2006) avaliaram a próstata de ratos neonatos expostos a altos níveis de estrógeno, onde observaram desregulação entre o receptor de estrógeno alpha ($ER\alpha$), receptor de estrógeno beta ($ER\beta$), receptor de andrógeno (AR) e receptores retinoides (RARs). Além disso, houve alteração da expressão de morfogenos expressos no mesênquima prostático, assim, concluiu que altas doses de estrógeno em períodos de desenvolvimento estão relacionadas com alterações estruturais e funcionais na próstata que podem persistir ao longo da vida, causando assim o *imprinting* estrogênico (PRINS et al., 2006). Para que seja desencadeada uma resposta fisiológica, o estrógeno atua em receptores específicos, os receptores nucleares de estrógeno (ERs). Há dois membros na família dos ER's, o $ER\alpha$ e $ER\beta$, que são moléculas distintas, possuindo estrutura e peso moleculares diferente, sendo o $ER\beta$ ligeiramente menor que o $ER\alpha$ (JIA; DAHLMAN-WRIGHT; GUSTAFSSON, 2015). Na próstata a localização desses receptores varia, de acordo com o processo fisiológico desencadeado, o $ER\alpha$ está localizado nas células estromais enquanto o $ER\beta$ nas células epiteliais (PRINS et al.,

1998; PRINS; BIRCH, 1997). Os receptores desempenham papéis antagônicos na próstata, sendo o ER α , associado a processos de proliferação celular, enquanto o ER β desempenha papel antiproliferativo (DA; LU; WANG, 2015; LATIL et al., 2001; STETTNER et al., 2007; ZHAO; ZHOU; GUSTAFSSON, 2019).

A exposição a concentrações anormais de testosterona durante a gestação é responsável por alteração na organogênese prostática e no padrão de marcação positiva para o AR, ER α e em marcadores de proliferação celular na próstata de gerbilos machos e fêmeas ao nascimento (RAMOS et al., 2020). Gomes e seus colaboradores (2020), demonstraram que a próstata de gerbilos, ao serem expostos ao alumínio na vida pós-natal, apresentavam um aumento nas concentrações séricas de testosterona e da imunomarcação de células ER α positivas, associando esses resultados a um aumento da proliferação de células epiteliais e estromais, bem como um aumento do compartimento epitelial e diminuição do estroma (GOMES et al., 2020). A partir de dados clínicos de pacientes com CaP, Shen e seus colaboradores (2019) estabeleceram a que a marcação positiva para integrina $\alpha 6$ (CD49f) e ER α se correlacionavam com o fenótipo de células troco do CaP, e que apresentavam maior potencial metastático de transição epitélio-mesênquima (SHEN et al., 2019). Além disso, o ER α atua em processos inflamatórios e no estabelecimento de lesões pré-neoplásicas (BONKHOF, 2018).

Camundongos ER β $^{-/-}$, apresentam maior taxa de proliferação celular, de células basais (p63 $^{+}$), redução de moléculas pró-apoptóticas e ao envelhecerem desenvolvem lesões hiperplásicas na PV, com aumento da presença de infiltrados inflamatórios (IMAMOV et al., 2004). Dados do transcriptoma desses animais ER β $^{-/-}$, demonstraram aumento da expressão de moléculas pró-inflamatórias e inibidores apoptóticos, com uma regulação negativa de enzimas antioxidantes, diminuição de genes supressores tumorais e do *Pten* (WU et al., 2017). Estudos com células prostáticas benignas, da linhagem PNT1A, mostraram o que antagonista de ER β é capaz de modular positivamente a expressão de enzimas antioxidantes frente a resposta à Zearalenona, uma micotoxina mimética ao estrógeno (KOWALSKA et al., 2019). Células tumorais, PC3 e DU-145, com super expressão de ER β , ao serem expostas a condições de estresse apresentavam diminuição da resposta inflamatórias e de vias de proliferação celular (NF κ B), sugerindo seu papel como agente anti-inflamatório e anticâncer (XIAO et al., 2019).

O estrógeno ainda possui receptores não genômicos, o receptor estrogênico acoplado a proteína G, o chamado GPER1/GPR30 (LEVIN, 2015; PROSSNITZ et al., 2008; SHARMA; PROSSNITZ, 2017). Apesar de terem sido descritos recentemente, muitos trabalhos vêm citando a participação desse receptor na resposta estrogênica, tanto na próstata como em outros órgãos, como pâncreas, fígado, músculo estriado esquelético, tecido adiposo e cérebro (SHARMA; PROSSNITZ, 2017). Estudos experimentais descreveram que a ativação da GPER1 foi responsável por aumentar as concentrações intracelulares de Ca^{2+} , através da ativação da fosfolipase C e do Inositol-1,4,5-trifosfato (IP3), levando essas células à apoptose e ao desenvolvimento da HPB (YANG et al., 2017). Estudos com fibroblastos normais, mostraram que a super expressão do GPER1 leva essas células a limitar a via $\text{ER}\alpha$, através de inibição o AMP cíclico/Proteína quinase A (AMPC/PKA), dando a essas células um fenótipo de fibroblastos associado ao CaP (JIA et al., 2016).

1.5 Epigenética, Programação Fetal e Estrógeno

Compreender e elucidar os mecanismos epigenéticos é um dos maiores desafios para compreender as respostas fenotípicas desencadeadas ao *DOHaD* (GOYAL; LIMESAND; GOYAL, 2019; WADHWA et al., 2009). Apesar desses mecanismos epigenéticos serem bem explorados sob a ótica do desenvolvimento embrionário e da carcinogênese, os dados associados a *DOHaD* e RPM ainda são escassos. O que torna particularmente importante, uma vez que a janela de programação torna o embrião/feto susceptível a diversos fatores, como a nutrição materna e a toxicologia ambiental, impactando nos níveis de expressão gênica, metilação de regiões promotoras no DNA e expressão de microRNAs (HEERWAGEN et al., 2010; HO et al., 2017; KELEHER et al., 2018; SCARANO et al., 2019).

Durante a década de 1940 o biólogo Conrad Waddington cunhou o termo epigenética, ao unir epigênese (estudo do crescimento e diferenciação embrionário) e genética (WADDINGTON, 1940). Com o passar do tempo e a descoberta de novos conhecimentos e técnicas de biologia molecular, a definição de epigenética foi evoluindo e incorporando-os. Skinner e seus colaboradores (2010) definem epigenética como “Fatores e processos moleculares ao redor do DNA que são mitoticamente estáveis e regulam a atividade do genoma independentemente da sequência do DNA”, que engloba as modificações de histonas, a metilação do DNA, alteração na estrutura da

cromatina e a expressão de RNAs não codificantes (ncRNA) (SKINNER; MANIKKAM; GUERRERO-BOSAGNA, 2010).

Os ncRNAs são uma classe heterogênea de moléculas de RNA que tem despertado interesse científico nos últimos anos, principalmente por atuar na regulação de diversos processos biológicos, como expressão gênica, inativação de um dos cromossomos X e de elementos transponíveis (GUTTMAN; RINN, 2012; LODA; HEARD, 2019; OZATA et al., 2019). Uma das classes mais estudadas dos ncRNA, são os miRNAs/microRNAs, uma superfamília de ncRNA, que possuem aproximadamente 22 nucleotídeos e que medeiam o silenciamento gênico, sendo um importante regulador pós-transcricional, os miRNAs estão envolvidos em quase todos os processos celulares sendo essencial para o desenvolvimento embrionário, diferenciação celular, homeostase e no surgimento de desordens (GEBERT; MACRAE, 2019; KROL; LOEDIGE; FILIPOWICZ, 2010).

Os miRNAs podem ser derivados de regiões intrônicas, ou a partir de miRgene, esses últimos são transcritos por uma RNA polimerase II, dando origem ao pri-miRNA, um complexo proteico que checa a molécula e cliva as caudas 5' e 3' de suas extremidades, formando os pré-miRNAs (~70 nucleotídeos), e com o auxílio de exportinas são levadas ao citoplasma. No citoplasma a RNA polimerase III (Dicer) juntamente com proteína de ligação ao RNA TAR (TARBP) e pelo ativador de proteínas da proteína quinase induzida por interferon (PACT), clivam a extremidade do pré-miRNA, uma das fitas é descartada enquanto a outra se liga a proteína argonauta, o complexo miRNA-Argonauta se ligam as fitas de mRNA por complementariedade e pode levar a uma degradação da molécula de mRNA ou simplesmente impedir sua tradução (GEBERT; MACRAE, 2019; KROL; LOEDIGE; FILIPOWICZ, 2010). Na Figura 5 está representado o processo de biogênese e os mecanismos de atuação dos miRNAs.

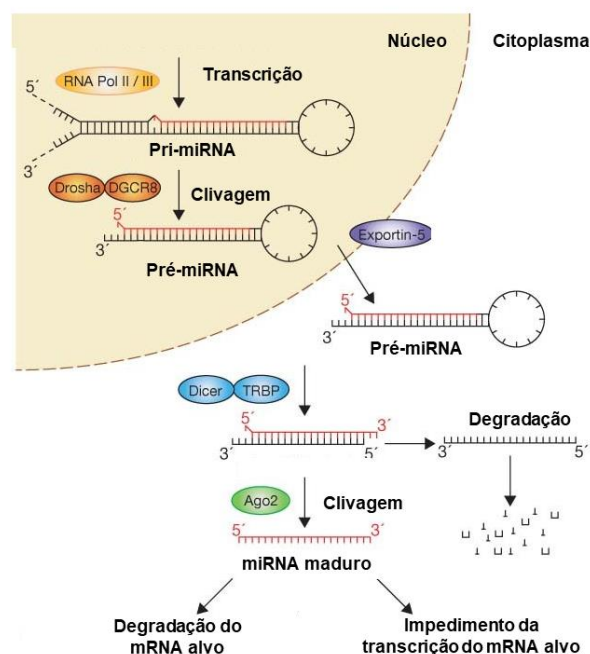


Figura 5. Esquema representativo da biogênese e mecanismos de atuação do miRNA, imagem adaptada de WINTER et al., 2009.

Através de ferramentas de bioinformática, é permitido encontrar quais mRNAs são alvos dos miRNAs. A maioria das plataformas de bancos de dados para análise de miRNA, usam refinados algoritmos para gerar seus resultados, e a maioria deles divide seus resultados em alvos preditos e validados. Sendo os alvos preditos aqueles que apresentam complementariedade entre o mRNA e o miRNA, e os alvos validados são aqueles que através de experimentação comprovaram a interação entre o miRNA-mRNA (GEBERT; MACRAE, 2019; STICHT et al., 2018). Recentemente, alguns trabalhos descrevem que há uma modulação da expressão de miRNAs e genes por eles regulados, frente a variações dos níveis estrogênicos, mostrando que há participação dos estrógenos nas regulações epigenéticas (KLINGE, 2015). Através de dados clínicos de pacientes com câncer de mama depositados no *The Cancer Genome Atlas* (TCGA), foi possível estabelecer diferentes padrões de expressão de miRNA e mRNA em pacientes ER⁺ e ER⁻, tendo esses alvos como possíveis marcadores de pior prognóstico (XIAO et al., 2018). Scarano e seus colaboradores (2019) demonstraram que ratos submetidos a ftalatos durante o desenvolvimento intrauterino apresentavam alteração no padrão de expressão dos miRNAs na PV (SCARANO et al., 2019).

2. Justificativa e relevância do tema

Dada a importância da próstata a homeostase do sistema genital masculino e o sucesso reprodutivo, a próstata mostra-se interessante do ponto de vista clínico, uma vez que ela é acometida por uma grande incidência de lesões, tais como HPB e CaP (DASGUPTA et al., 2012). Segundo Willian Gardner o surgimento do CaP está associado a variações na concentração estrogênica no ambiente intrauterino (GARDNER, 1995). Estudos experimentais demonstraram que os elevados níveis de estrógeno em fases críticas do desenvolvimento causam um *imprinting* estrogênico, reprogramando a glândula para uma resposta exacerbada ao estrógeno ao longo da vida com consequente aumento nas desordens prostáticas no envelhecimento (PRINS et al., 2006; SANTOS et al., 2019). Além de alterar o desenvolvimento prostático, homeostase glandular e o padrão de expressão dos miRNAs (PRINS; KORACH, 2008; SANTOS et al., 2019; SCARANO et al., 2019). Dessa forma, dada à importância dos hormônios esteroides, em especial o estrógeno e a testosterona, e seu papel no ambiente intrauterino para a morfogênese e homeostasia prostática. Parece-nos fundamental identificar as alterações do perfil esteroidogênico da prole de ratos submetidos a RPM, bem como compreender o papel dos miRNAs e mRNAs na regulação molecular dessas vias de sinalização intraprostáticas.

3. Objetivos

3.1 Objetivo geral:

Avaliar os efeitos da RPM gestacional e lactacional sobre a síntese sistêmica dos hormônios esteroides, correlacionando a contribuição da Próstata Ventral (PV) e dos tecidos periféricos, de ratos machos *Sprague Dawley* no DPN21. Caracterizando os níveis de hormônios esteroides e seus metabólitos intermediários, a expressão genica de enzimas da via bioquímica esteroidogenica, o papel dos receptores androgênicos e estrogênicos, bem como a participação de miRNAs e mRNAs estrógeno responsivos.

3.2 Objetivos específicos:

Usando animais de 21 DPN submetidos a RPM durante a gestação e lactação objetivamos:

- Quantificar os níveis séricos de colesterol total e dos hormônios esteroides, pregnenolona, progesterona, desidroepiandrosterona (DHEA), testosterona e estrógeno, de forma sistêmica (sangue);

- Investigar a expressão genica de enzimas: aromatase (*Cyp19a1*), 5 α Redutase (*Srd5a3*), Cyp450scc (*Cyp11a1*), Cyp7 (*Cyp7b1*), 3 β HSD (*Hsd3b7*), 17HSD (*Hsd17b6*) responsáveis pela síntese de hormônios estrogênicos na PV, fígado, adrenal, testículo e pele;

- Analisar a expressão gênica dos Receptor de Andrógeno (*Ar*), Receptor de Estrógeno alpha (*Esr1*), Receptor de Estrógeno beta (*Esr2*) e Receptor de Estrógeno acoplado a proteína G (*Gper1*) na PV;

- Realizar análise integrativa dos miRNAs diferencialmente expressos (DE) com os mRNAs DE;

- Validar a expressão do *miR206-3p* na PV, fígado, testículo, adrenal e pele;

- Avaliar os efeitos da superexpressão do *miR206-3p* em células prostáticas benignas (PNT-2);

- Associar o papel dos alvos preditos ao *miR206-3p* com pacientes com câncer de próstata, a partir de análises *in silico*.

4. Resultados

Os resultados obtidos por esse trabalho encontram-se disponível no Capítulo 2 em formato de artigo intitulado “*Maternal malnutrition alters steroid biosynthesis and increase the expression of tumor suppressor miR206-3p in the ventral prostate of young rat offspring*”.

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Capítulo 2. Artigo Científico

Maternal malnutrition alters steroid biosynthesis and increase the expression of tumor suppressor miR206-3p in the ventral prostate of young rat offspring

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Highlights

- MLPD alters the steroidogenic profile in young offspring rats;
- MLPD leads to hormonal imbalance and deregulation of normal prostate development;
- Maternal malnutrition deregulated the prostatic expression of AR, ER α and GPR30

mRNAs in young rat offspring;

- miR206-3p-mRNAs network may be involved in the prostate response to maternal exposure to low protein diet.

Abstract

A maternal low protein diet (MLPD) is a determining factor in altering the health of offspring throughout life, placing it within the approach of the Developmental Origin of Health and Disease (*DOHaD*). It is associated with MLPD changes in serum levels of steroid hormones, loss of normal prostate development and becomes a determining factor for the increased incidence of prostate cancer (PCa) with aging. Here we describe that animals on a postnatal day (PND) 21 and that submitted MLPD, had low birth weight, decreased serum levels of progesterone, dehydroepiandrosterone, increased cholesterol, testosterone, and estrogen. We associate the increase in these hormones, especially estrogen, with the increased expression of *Cyp19a1* (aromatase) in the skin, liver, and adrenal gland. As a result of this increase in estrogen levels, we observed a loss of normal development of the ventral prostatic (VP) lobe leading to smaller glands. Studies of miRNA sequencing showed the miR206-3p upregulated in the VP of these animals, through in silico analyzes we identified that the targets predicted to miR206-3p are potentially contributing to prostatic carcinogenesis, these targets lead to worse prognostic of survival in patients human with PCa. Assays that mimic the expression of miR206 in human prostatic cells normal (PNT2 lineage) showed a reduction in the cell viability as a result of an increase in miR206-3p.

Key words: *DOHaD*; maternal low protein diet; steroidogenesis; ventral prostate; miRNA

Introduction

In last decades, the incidences of metabolic syndromes and aging-related diseases have increased, becoming a public health problem worldwide. Many of these diseases are associated with a sedentary lifestyle, consumption of ultra-processed, low-nutritious, high fat/caloric diet, and also exposure to endocrine disruptors spread throughout the environment. Recently, early life exposure to adverse conditions have also been associated with metabolic reprogramming during intrauterine development with subsequent development of non-communicable chronic diseases with aging [1–3].

The search to understand and elucidate the origin of these diseases in adulthood and aging with the insults suffered during intrauterine development and/or early childhood fit the conception of Developmental Origin of Health and Disease (DOHaD) [4–6]. A theory that emerged more than thirty years ago, in the light of the English epidemiologist David Barker, who in a series of studies associated diseases in adulthood such as hypertension, diabetes and heart disease with individuals who were pregnant during the period of “Dutch Hunger Winter” [7–9].

Rodent model exposed to low protein diet during pregnancy and/or lactation has been used to elucidate the physiological and molecular mechanisms involved in the DOHaD outcomes [10, 11]. Using these models, it has been demonstrated offspring lower birth weight [12, 13], metabolic dysfunction [12–15], increased incidence of inflammatory process [16], and unbalance of steroid hormones [12, 17, 18]. Maternal exposure to low protein diet also directly affects liver [19], kidney [20], lung [21], heart [22] morphophysiology, as well as reproductive organs such as breast [23], ovary [24], epididymis [25, 26], and prostate [12, 13].

Regarding to prostate gland, recent data described that maternal malnutrition negatively affected the angiogenesis and overall ventral prostate growth (VP) at

postnatal day (PND) 10 and 21 [13], leading to the increased incidence of prostatic disorders with aging, including *in situ* carcinoma. These results were associated with unbalance of circulating steroid hormones, mainly increase of estrogen and reduction of testosterone throughout life span in maternally-malnourished offspring [12].

Biosynthesis of steroid hormones involves enzymes belonging to two classes, the cytochrome P450 monooxygenases (CYPs) and the hydroxysteroid dehydrogenases (HSDs) in a sequential reactions converting cholesterol to active hormones, such as pregnenolone, progesterone, DHEA, testosterone, and estrogen [27, 28]. Besides expressed in testis and ovary [29], these enzymes are also expressed in prostate [30], adrenal [31], skin [32], and liver [33]. As such, deregulation in tissue expression of these enzymes may lead to a systemic unbalance of steroid hormones and organ-specific response.

Although the importance of prostatic fluid for reproductive success, prostate cancer is the second diagnosed cancer in older men. Globocan estimated approximately 1.8 million new cases were diagnosed in 2018, leading to 360 thousand deaths [34]. Over the past decade, there is growing evidence associating prostate carcinogenesis with early-life exposure to an estrogenic environment [35, 36]. Epidemiological studies demonstrated the relationship between intrauterine exposure to high levels of estrogen and the increased incidence of prostate cancer in African-American men compared to Caucasians [37, 38]. The high levels of estrogen in critical periods of prostate development are associated with delay in prostate growth [39], changes in the regulation of key molecular signaling pathways involved in both normal prostate development [40], as well in the early stages of prostate carcinogenesis [41]. DOHaD, estrogen, and PCa are capable of altering epigenetic mechanisms of the prostate gland, including the pattern of miRNA expression [42, 43]. Scarano et al, demonstrated endocrine disruptors

that mimic estrogens, such as bisphenol A and/or phthalates, are responsible for morphological changes in the prostate and for altering the expression pattern of miRNAs [44].

In recent years, Santos et al. demonstrated, experimentally, that maternal malnutrition is a determining factor related to the increased incidence of PCa in older rat offspring, [12]. In a recent study, Santos et al., using the same rodent model, identified the key deregulated molecular signaling pathways related to the prostate carcinogenesis. Among them, growth factors, estrogen signaling, detoxification, and energy metabolism appeared deregulated in the prostate of both young and old rats [45], highlighting that importance of maternal nutritional status for maintenance of offspring health. Given that the role of steroid hormones for prostate morphophysiology and health, little is known about the steroidogenic profile in male offspring submitted to maternal malnutrition and the epigenetic mechanisms that can contribute to prostatic carcinogenesis.

Materials and Methods

Animals

Adult Sprague Dawley rats, females (90 days age, n = 30) and males (90 days age, n = 10) were used. These animals were subjected to the harem system (1 male to 3 females) mated overnight. After the determination of pregnancy on gestational day 1 (GD1), pregnant rats were distributed into two experimental groups (n=15/group): Control (CTR): Dams fed with a normal protein diet (17% protein) and Gestational and Lactational Low Protein (GLLP): Dams fed with a low protein diet (6% protein) during gestational and lactational periods. The normal and hypoprotein diets (LPD) followed the AIN-93 standards described by Reeves et al (1993) [46] and were provided by PragSoluções (PragSoluções, SP, Brazil). All diets were isocaloric and normosodic

(Supplementary Table 1) and were used in previous studies [12, 13, 18, 45]. To maximizing lactation performance, litters were reduced to eight pups at PND1: four males and four females[12, 47]. The rats were euthanized at postnatal day (PND) 21 (n=15/group) by a decapitation, it was weighing and the blood, Ventral Prostate (VP), testis, adrenal, liver, and skin were collected and processed by a different analysis as described below. The animal procedures were approved by the Biosciences Institute/UNESP Ethics Committee for Animal Experimentation (Protocol #1178) and were in accordance with ethical animal research principles and the Brazilian legislation established by the Brazilian Council of Control in Animal Experimentation.

Serum blood hormonal analysis

Blood samples from male offspring (n = 12/group) were centrifuged (2400 g for 20 min), and serum were used to determine the quantifications of total cholesterol (Labtest®, R76-2/100, Brazil, sensitivity: 0.06 mg/dL), pregnenolone (LifeSpan BioSciences®, LS-F39295, USA, sensitivity: 9.375 pg/ml; samples were diluted 1:10), dehydroepiandrosterone (DHEA) (Monobind®, CA 7425-300, USA, sensitivity: 0.10 ng/ml), progesterone (Monobind®, CA 4825-300, sensitivity: 0.105 ng/mL), estrogen (17 β estradiol, Monobind®, CA 4925-300, USA, sensitivity: 6.5 pg/mL) and testosterone (17 β -hydroxy-4-androstene-3-one, Monobind®, CA 3725-300A, USA, sensitivity: 0.038 ng/mL). The hormonal qualifications were made in 96-well plates using the ELISA plate reader (Epoch™, Biotek Instruments, VT, USA) following the manufacturers' protocol.

Morphological analysis

Samples of VP from CTR and GLLP groups (n = 06/group) on PND21 were fixed for 4 h in Methacarn (70% methanol + 20% chloroform + 10% acetic acid),

dehydrated in ethanol, diaphanized in xylene, and embedded in Paraplast (Sigma). Histological sections (05 µm) were stained with Hematoxylin/Eosin for general morphological analysis.

Identification of deregulated miRNAs and mRNA targets

We applied Next Generation Sequencing (RNAseq) to characterize the microRNome and transcriptome profile in the VP of young rat offspring (PND21) submitted to MLPD in gestational and lactation periods. We demonstrated several miRNAs and targets mRNA deregulated in the VP of these animals. The followed criteria were used for the selection of miRNA were: a number of reads in the miRNome, higher fold change, and the number predicted targets, p-value < 0.05, and Log₂ Fold Change < 0.6. In silico analysis of prediction for target mRNAs potentially modulated by miR deregulated were performed using miRWalk 3.0 (<http://mirwalk.umm.uni-heidelberg.de/>) [48] for *Rattus norvegicus*. We considered as a predicted target those that had free energy of binding ≤ -20 [49]. After that, we used our transcriptome database to integrate and identify shared predicted and mRNAs deregulated in transcriptomic analysis, take into account the correlation of upregulated miRNA with downregulated target mRNA. After analysis, we selected the miR206-3p to deeper investigate its role in prostate response to maternal malnutrition.

Was analyzed to identify the potential of miRNA206-3p to be secreted. This analysis was conducted using the information on extracellular and circulating non-coding RNAs through miRandola database (<http://mirandola.iit.cnr.it/index.php>), database of extracellular circulating miRNAs [50].

Characterization of the participation of miR206-3p targets in human PCa: in silico approach

To give further translational insights into the relationship between targets of miR206-3p with those from patients with PCa, we identified differentially expressed genes between normal human prostate tissue and human PCa samples. This set of differentially expressed genes was compared to the targets of miR206-3p (ZMAT3, PLG, RIMBP2, ZNF366, DTNA, LHFPL2 and PBLD) identified in our samples. The SurvExpress database (<http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp>) was used for survival analysis and risk assessment in the Prostate Adenocarcinoma dataset patients (PRAD-TCGA). This tool allowed the association between the expression of the molecular markers identified in prostate carcinoma in situ with the survival of patients with PCa using Cox Proportional Risk Regression, according to risk groups estimated by an optimization algorithm. Additionally, we used The Human Protein Atlas (HPA) (<https://www.proteinatlas.org/>) database to demonstrate the distribution and localization of these proteins in normal and tumor samples by immunohistochemistry.

Cells and culture conditions

PNT2 benign prostatic epithelial cell lines were used for analysis. The cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC). PNT2 cell lines were cultured using RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FBS, 50 µg/mL penicillin-streptomycin and 0.5 µg/mL amphotericin B (GIBCO / Invitrogen). The cells were grown at 37°C with 5% CO₂ in a humid atmosphere. The culture medium was changed twice a week and, throughout the experimental phase, the cells were monitored with the aid of an inverted microscope

(Zeiss Axiovert). For passage, the cells were released with 0.25% trypsin (GIBCO/Invitrogen) for 5 min at 37 ° C, resuspended in growth medium and plated again.

Oligonucleotides and transfection

The miR-206-3p mimic (Thermo Fisher, mirVana™ miRNA Mimic, code: 4464066; MC10409) and its negative control (Thermo Fisher, mirVana™ miRNA Mimic Negative Control) before transfecting the cells formed a complex with the reduced serum medium Opti-MEM (Thermo Fisher Scientific, USA). Transfections were performed in PNT2 cells with RNAiMAX lipofectamine (Thermo Fisher Scientific, USA) combined with 10 nM of each oligonucleotide for 16 hours, when the cells were approximately 80% confluent, whether treated with the RPMI medium. The total mRNA of the samples was extracted with TRIzol (Thermo Fisher Scientific, USA) as recommended by the manufacturer.

Cell viability and proliferation - MTT assay

Cell viability was be determined by the MTT reduction method (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazoline bromide). PNT-2 cells were seeded, in 24-well plates at a cell density of 8×10^4 in 500µl of complete RPMI medium per well. After proliferation for 24, 48 and 72 hours, the media was being exchanged with RPMI medium (without fetal bovine serum) containing 10% MTT. The cells were be incubated for 4 hours at 37 ° C. After this period, the medium was be removed and 600µl of DMSO will be added to the wells, which, after homogenization, will be transferred (3x 200µl) to 96 well plates for reading. The microplate will be read with 550nm absorbance to determine the cell proliferation indexes, which are directly proportional to the colorimetric intensity.

Preparation of cDNA from mRNA

The RNA was extracted according to Trizol manufacturer's instructions (Thermo Fisher) and treated with TURBO DNA-free Kit (Thermo Fisher #AM1907). Aliquots of 2 µg of total RNA of VP, testis, adrenal, brain, skin, liver rats, were reverse transcribed using the High-Capacity Kit RNA-to-cDNA (Life Technologies), in 10 µl of reaction of according to the manufacturer's instructions. The cDNA was then diluted 25x with ultra-pure water and stored at -20 °C until use. Aliquots of cDNA from each sample were added to a mixture of reagents containing primers "sense" and "anti-sense" and the volume was completed to 10 µl with ultrapure water. Primers were designed specifically for target genes of interest after *in silico* filtering and the sequences are shown in Supplementary Table 2. The values obtained for all samples were normalized by the ratio between the informational gene and the reference gene (*Gapdh* and *Gusb*). The values were calculated using the expression ratio of the GLLP/CTR groups.

Preparation of cDNA from miRNA

First, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The RT-qPCR analysis was performed according to the relevant literature [51]. Basically, the RT primers stem-loop, designed according to CHEN et al. (2005)[52], was hybridized with the selected miRNA molecule, and then the reverse transcription reaction was performed. The reaction product was amplified using specific primers and SYBR Green PCR Master Mix system (Thermo Fisher, USA). The values obtained for all samples were normalized by the reference miRNA (U6). The values were calculated using the expression ratio of the GLLP/CTR groups. Knowing the potential of this miR to be secreted in the form of an

exosome and to be found circulating in the blood, we investigated its expression in other organs, such as adrenal, liver, skin and testis.

Analysis of gene expression by RT-qPCR

In both cases, the expression of mRNAs and miRNAs, were performed in duplicates for each target gene in the Real Time QuantStudio 12K flex System (Applied Biosystems) on 96 plates wells according to the manufacturer's instructions. The relative quantification of each gene was performed using the $2^{-\Delta\Delta CT}$ method[53]. All information about the primers is in Supplementary Table 2.

Statistical analysis

Statistical tests were performed using the GraphPadPrism® software (version 5.00, Graph Pad, Inc., San Diego, CA). The results were submitted to normalization analysis (Shapiro-Wilk test). Then, the data which passed in this test were compared using a t-test. The remaining were submitted to the Mann-Whitney. Data were expressed as mean $\pm$ SD. The differences were considered statistically significant when $p < 0.05$.

Data representation and analyses

The results of gene expression in this work are represented in bar graphs that were constructed using the GraphPad Prism tool (GraphPad Software). To demonstrate the interaction networks between the miRNA-mRNAs, circus plot graphics were used, which were generated in the environment R using the “Circlize” package[54]. The Hetmap with the results of RT-qPCR was created using the Morpheus web tool (<https://software.broadinstitute.org/morpheus>).

Results

Biometric parameters and hormonal analysis

Dams from GLLP group had a lower body weight gain during pregnancy and lactation (CTR 135.50 ± 18.28 vs GLLP 106.65 ± 20.62), although there were no changes in diet consumption (CTR 18.74 ± 1.32 vs GLLP 20.06 ± 2.73) and energy intake (CTR 310.30 ± 22.01 vs GLLP 326.20 ± 61.38). Male offspring from GLLP group presented lower body weight compared to the CTR group on both PND1 and 21 (CTR 7.06 ± 0.66 vs GLLP 5.91 ± 0.56 ; CTR 45.82 ± 5.74 vs GLLP 16.7 ± 1.92 , respectively) (Figure 2A-B). The anogenital distance (AGD) was reduced in both GLLP groups on PND 1 (CTR 3.30 ± 0.39 vs GLLP 2.88 ± 0.47) and PND 21 (CTR 8.77 ± 1.39 vs GLLP 5.61 ± 0.64) compared with CTR groups. The VP weight were lower in GLLP PND21 to compared CTR group ($0.02 \pm 0.0083 > 0.0121 \pm 0.0021$). The serum levels of total cholesterol, estrogen (17β -estradiol) and testosterone (17β -hydroxy-4-androstene-3-one) increased in GLLP group compared to CTR group (CTR 158.7 ± 25.35 vs GLLP 792.9 ± 183.9 ; CTR 15.69 ± 1.02 vs GLLP 20.32 ± 3.05 ; CTR 0.21 ± 0.12 vs GLLP 0.38 ± 0.16 , respectively), while a decrease in pregnenolone (CTR $6,809 \pm 1,511$ vs $5,085 \pm 1,072$) dehydroepiandrosterone (DHEA) (CTR 16.22 ± 9.02 vs GLLP 0.38 ± 0.61) and progesterone (CTR 19.98 ± 10.96 vs GLLP 11.69 ± 6.02) occurred in GLLP group compared to the CTR group. These results were summarized in the Table 01.

The steroidogenic profile

Increased *Cyp11a1* gene expression was observed in VP and skin, with reduction in testis and liver. *Hsd3b7* had its genetic expression increased in VP. *Hsd17b6* expression was increased in VP, adrenal and skin by a reduction in the liver

and testis. *Srd5a3* was increased in VP with decreased expression in the testis and adrenal. *Cyp19a1* increased its expression in the adrenal, skin and liver with a reduction in the testis and VP. The *Cyp7b1* was decreased in the PV, testis and adrenal (Figure 1 and Supplementary Figure 1-6).

Alterations prostatic morphology and hormones response intraprostatic

Morphological analysis demonstrated impairment of VP growth the GLLP group, which presented reduced acini and increase in the stromal compartment when compared to the CTR group (Figure 2C-D). In addition, we can observe a decrease in the gene expression of the Estrogen Receptor *alpha* (*Ers1*) and G Protein-Coupled Estrogen Receptor 1 (*Gper1*), in addition to an increase in the androgen receptor (*Ar*) (Figure 2E-H).

In silico analysis

The comparison of microRNA profile between CTR and GLLP groups identified 20 miRNAs differentially expressed (DE), 14 up-regulated and 6 down-regulated (Supplementary Table 3) in the VP. The transcriptome identified 708 mRNA DE, 525 up-regulated and 183 down-regulated (Supplementary Table 4). Target prediction analysis indicated that the 20 miRNA DE had 24,263 mRNAs as predicted targets (Supplementary Table 5). The predicted mRNA data were compared with the VP DE mRNA, resulting in 90 mRNA regulated by 17 miRNAs (Figure 3A and Supplementary Table 6). Of these 17 miRNAs, 14 of them are secreted out of cells through Argonaut2 protein, microvesicle, exosome, and other circulating forms, the latter two being more common (Table 2). Finally, we arrived at miR-206-3p to follow validations; it presented the highest Fold Change 1.79 (Log_2), was found outside the cells and had a good number of predicted DE targets in the VP of the animals at PND21. The predicted genes

were *Zmat3*, *Plg*, *Rimbp2*, *Znf366*, *Dtna*, *Lhpl2*, and *Pbld* and its main functions are described in Table 3.

The expression of miR206-3p in the VP and other organs

The results of gene expression showed that *miR206-3p* was up-regulated in the animals' VP, confirming the sequencing data (Figure 3C). We can also observe the increase in its expression of *miR206-3p* in the liver, adrenal, and testis. The expression in the skin not changed (Figure 3D-G).

In silico analysis confirmed the relationship between miR206-3p targets and human PCa

To better understand how the 7 predicted targets *miR206-3p* behaved in prostatic tumorigenesis, we investigated whether they would be able to predict a worse prognosis, impacting the survival of these patients, these analyzes were generated in the tool SurvExpress (Figure 4A). These genes were able to divide the 497 patients with PCa into two distinct groups, those with high (red line) and low risk (green line). In the survival curve, we can see that the presence of these genes prescribed a significant reduction in life expectancy (hazard ratio (HR) = 9.81; log-rank p-value = 0.000478). The heatmap shows separately the behavior of each gene in the groups with the highest and lowest risk (Figure 4B). It was possible to observe that ZMAT3, RIMBP2, ZNF366, and LHFPL2 showed a reduction in their expression in high-risk patients, the DTNA and PBLD were shown to be increased, while the PLG was overexpressed in both groups.

We identified human tissue immunohistochemistry in HPA for 6 of the predicted targets (ZMAT3, PLG, ZNF366, DTNA, LHFPL2, and PBLD) for *miR206-3p* (Figure

4C). Analyzing the immunostaining of normal and tumor tissues, we could observe that there was a decrease in ZMAT3, PLG, ZNF366, and LHFPL2 in the tumor regions when compared to normal tissue. While DTNA and PBLD showed no difference in their immunostaining. These results highlight the involvement of maternal malnutrition in the dysregulation of proteins involved in prostate tumors, with miR206-3p being a potential regulator of these pro-carcinogenic changes.

The effects in the overexpression of miR206-3p in the benign cell prostate

In order to understand the functional role of increased miR206-3p expression in the prostate of animals submitted to MLDP, we performed tests that mimic miR206-3p expression in benign prostatic epithelial cells, PNT-2 lineage. The cells subjected to the mimic test did not show any marked morphological changes (Figure 5A). Expression data shows that the mimic assay was successful, and there was an increase in miR206-3p relative expression with treatment (Figure 5B). The MTT assays showed that the increase in miR206-3p expression was associated with a reduction in the viability of prostate cells, after 48 and 72 hours of weaning (Figure 5C), when compared to control cells.

Discussion

Lower birth weight and lower weight gain at weaning may be associated with maternal consumption of low protein diet during gestation and lactation (Colombelli et al., 2017; Santos et al., 2019), and changes in the gene expression associated with fetus growth [55]. The AGD is an important biomarker of the response to androgens in the intrauterine period since it is regulated by testosterone and DHT produced by fetal testicles [56] and the reduction of this parameter may be associated with the reduction in the testosterone in these animals at PND10, which affects other organs in male genital

system, such as prostate, testis, and epididymis [13, 26, 57]. Taken together, these results showed that maternal malnutrition definitively alters the developmental biology of the progeny, with last long consequences across the life span.

The increase in serum levels of total cholesterol indicates that, although reduced weight in offspring submitted to maternal malnutrition, their metabolic status is altered. It has already been described that maternal malnourished dams show a reduction in visceral, retroperitoneal, and periepididymal fat deposits, in addition to an increase in serum levels triglyceride [12], changes in liver functions, mammary glands and alteration in the milk composition [58]. Pregnenolone is an intermediate metabolite for synthesis of steroid hormones, and its production occurs mainly in mitochondria. It is classified as a neurosteroid due the synthesis occurs mostly in the nervous system. Studies show that changes in serum pregnenolone levels are associated with neuroendocrine disorders and changes in neuroplasticity [59]. DHEA is one of the main steroid hormones produced mainly by the adrenal glands, being responsible for regulation of several metabolic processes, such as adipogenesis, the response to leptin and glucose metabolism, with DHEA being considered a protector against aging [60]. It has already been described that maternal malnutrition is responsible for reducing the size of the adrenals and for altering the tissue structure [61, 62]. Progesterone plays a central role in processes and mechanisms in male offspring. It is responsible for regulating the development of the central and peripheral nervous system, in addition to being associated with stress responses, aggressive behavior, the reduction in levels of progesterone were associated with a lower reproductive success [63, 64]. Testosterone is an important steroid hormone that regulates the development of the genital system [65, 66]. The literature reports that maternal malnutrition is responsible for altering the serum levels of these hormones at different times in the offspring life [12, 13]. High

levels of estrogen in male offspring is associated with alteration in the development process of the genital system, such as delay the development of the testicles, epididymis, and prostate [25, 26, 57, 67].

Pregnenolone, DHEA, and progesterone are precursor hormones in the steroidogenic pathway and may be metabolized to testosterone, which can then be converted into estrogen and DHT [28]. In this context, we believe that reduction in serum levels of intermediary metabolic molecules in male offspring submitted to maternal malnutrition can interfere with the development of steroid-responsive organs, such as those from the genitourinary system. In addition, we believe that these molecules may be used for conversion to testosterone, as an attempt to maintain the levels of this hormone in these animals. However, we believe that testosterone follows in the estrogenization process since there is a reduction in the expression of 5 alpha-reductase (*Srd5a3*) in the testicles and adrenal and an increase in the expression of aromatase (*Cyp19a1*) in large organs and with a great impact on the response metabolic, such as skin, liver and adrenal.

High levels of ACTH lead to an imbalance in the gene expression of steroidogenic enzymes, such as those from Cyps family, thus inhibiting the clearance of steroid hormones [68], and are associated with the development of adrenocortical carcinoma [69, 70]. Studies showed that the skin is classified as a steroidogenic organ independent of endocrine stimuli and that it has the potential to metabolize cholesterol, affecting the systemic levels of the steroidogenic metabolites, in addition to affecting the immune response of various organs and tissues [32, 71]. Studies with human fetuses have shown that the liver is essential for steroidogenesis in important stages for fetal/embryonic development [72]. Intratesticular steroidogenesis is susceptible to a reduction when exposed to molecules that mimic the action of estrogens, such as

bisphenol A (BPA) and tamoxifen, altering testicular growth by inhibiting the insulin signaling pathway and reducing the expression of enzymes that act on steroidogenesis [73, 74]. The VP of offspring rats submitted to maternal malnutrition showed an imbalance in the expression of steroidogenic enzymes. These results may be associated with the increased incidence of prostatic disorders, such as BPH and prostate cancer [75, 76]. When analyzing in a systemic way the expression of enzymes in these different organs, we believe that the increase in serum estrogen levels in maternal malnourished offspring is associated with the imbalance in the expression of steroidogenic enzymes in different organs, in particular by increasing the expression of *Cyp19a1* (aromatase) in the adrenal, liver, and skin.

In response to systemic hormonal variations, the VP presented abnormal development with a decrease of glandular fraction and increase in the stromal compartment [12, 13, 77]. Pregnant rats submitted to maternal malnutrition have high levels of circulating estrogen at gestation day 18.5 [78], and studies have demonstrated that exposure to estrogen during the critical window of prostate development alters epigenetic marks that favor the development of PCa with aging, a condition known as estrogenic imprinting [39, 79, 80]. The exposure to an estrogenized environment for a long period may favor the installation of a pro-inflammatory microenvironment and which after a chronic exposure can favor the onset of PCa [81, 82]. Thus, we believe that the prostatic changes observed in offspring submitted to maternal malnutrition are a consequence of the estrogenic imprinting suffered during intrauterine development, and the increase in Ar expression is an attempt by the gland to respond to testosterone and thus try to reverse the loss of prostate development.

miR206-3p has its role described by acting as tumor suppressor miRNA in several tumor types, including breast and prostate cancer, its function is associated with

regulation of epithelial-mesenchymal transition in tumor cells, in addition to inhibiting proliferation, migration, and the invasion, through activation of pathways associated with mTOR/PTEN/AKT cell proliferation; miR206-3p has been shown to have reduced expression in patients with PCa when compared to BPH [83–86]. Santos et al (2019) showed that the VP of offspring rats submitted to maternal malnutrition on PND21 showed a reduction in Akt protein expression, but an increase in p-Akt indicating its activation [12]. We believe that the increase in miR206-3p expression in the PV, adrenal, liver, and testis be associated response to the hormonal and metabolic unbalance observed in these animals.

The predicted targets indicate which possible cellular processes are being negatively regulated. We highlight the pathways associated with p53 (*Zmat3*), the regulation of angiogenesis (*Plg*), and the estrogenic response (*Zfp366*). We highlight the pathways associated with signaling p53 (*Zmat3*), the regulation of angiogenesis (*Plg*), and the hormonal response (*Zfp366*). Together, these signaling pathways show us an attempt by the prostate gland to alter its response to the hormonal imbalance environment, recover normal development and vascular architecture.

In conclusion, we describe how maternal low protein diet impacts male offspring steroidogenic profile, causing an imbalance in the levels of steroid hormones and leads to an increase in serum testosterone and estrogen levels. In response, VP changes the expression pattern of AR and ER receptors. Maternal malnutrition also regulates the expression of miR206-3p in VP and other organs that act in steroidogenesis. These epigenetic changes may be a possible consequence of the estrogenic imprinting during early life. We believe that increase in miR206-3p and the negative regulation of their targets may contribute to the increased incidence of PCa in these animals with aging.

Table and Figures

Table 1. Biometric parameters of the dams and offspring and hormonal levels at PND 21

Parameters	CTR group	GLLP group
Dams body weight gain in the pregnancy and lactation (g)	135.50 ± 18.28 ¹	106.65 ± 20.62 ²
Diet consumption in the pregnancy and lactation (g)	18.74 ± 1.32	20.06 ± 2.73
Energy intake of Dams during pregnancy and lactation (KJ/day)	310.30 ± 22.01	326.20 ± 61.38
Offspring weight at PND1 (g)	7.06 ± 0.66 ¹	5.91 ± 0.56 ²
Anogenital distance (AGD) at PND1 (mm)	3.30 ± 0.39 ¹	2.88 ± 0.47 ²
Offspring weight at PND21 (grams)	45.82 ± 5.74 ¹	16.7 ± 1.92 ²
Anogenital distance (AGD) at PND21 (mm)	8.77 ± 1.39 ¹	5.61 ± 0.64 ²
VP weight offspring at PND 21 (g)	0.02 ± 0.0083 ¹	0.0121 ± 0.0021 ²
Total cholesterol levels offspring at PND 21 (mg/dL)	158.7 ± 25.35 ¹	792.9 ± 183.9 ²
Pregnenolone levels offspring at PND 21 (pg/ml)	6,809 ± 1,511 ¹	5,085 ± 1,072 ²
Progesterone levels offspring at PND 21 (ng/ml)	19.98 ± 10.96 ¹	11.69 ± 6.02 ²
Dehydroepiandrosterone (DHEA) levels offspring at PND 21 (ng/ml)	16.22 ± 9.02 ¹	0.38 ± 0.61 ²
17β-hydroxy-4-androstene-3-one levels offspring at PND 21 (ng/ml)	0.21 ± 0.12 ¹	0.38 ± 0.16 ²
17β-estradiol levels offspring at PND 21 (pg/ml)	15.69 ± 1.02 ¹	20.32 ± 3.05 ²

g: grams. KJ= Kilojoule. mm= millimeter.

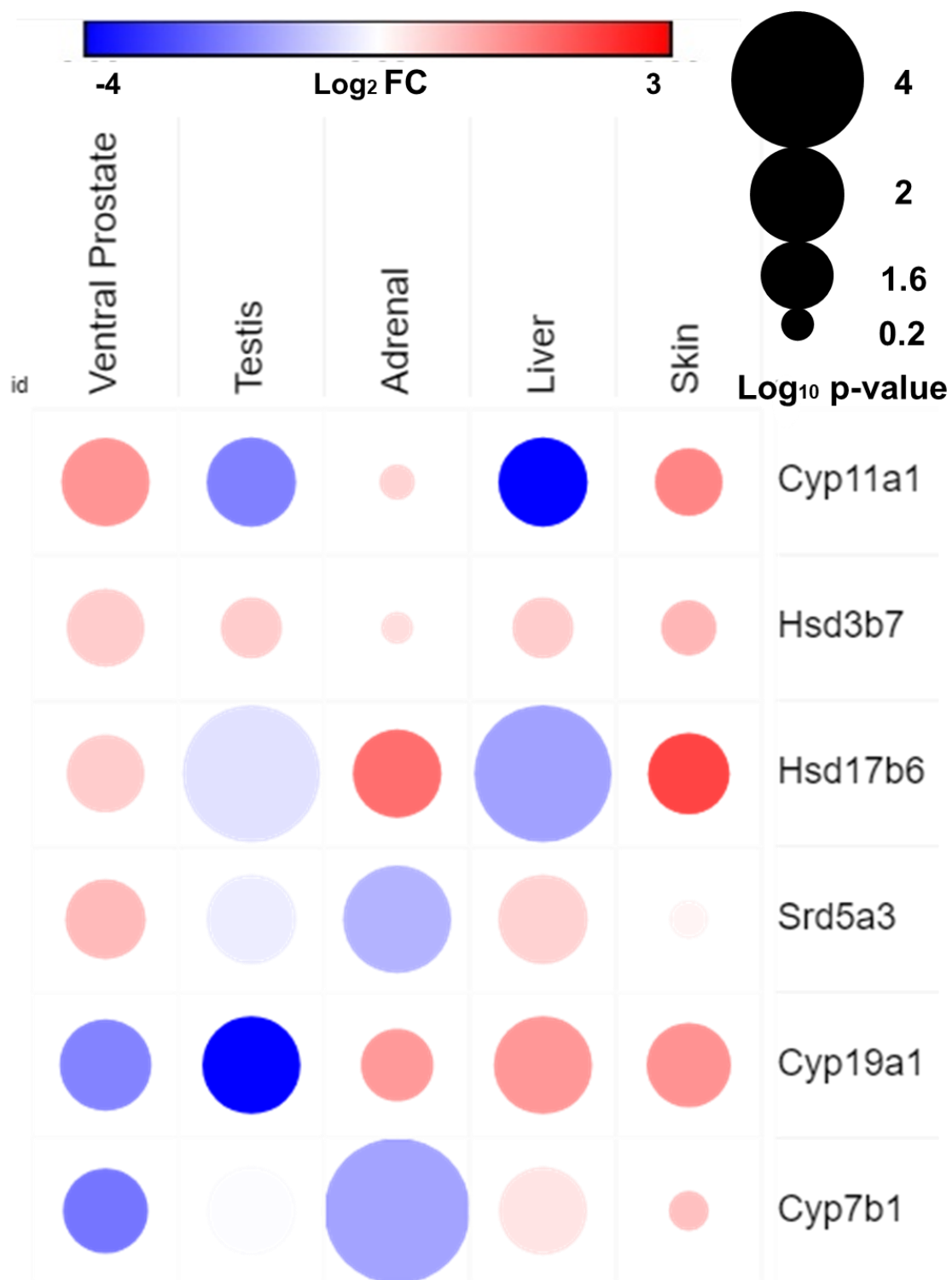


Figure 1. Representative image of the RT-qPCR result for enzymes that act in the steroidogenic pathway Cyp11a1, Hsd3b6, Hsd17b7, Srd5a3, Cyp19a1, and Cyp7b1. In ventral prostate, testis, adrenal, liver, and skin. The results were expressed in Log2 Fold Change e Log₁₀ p-value.

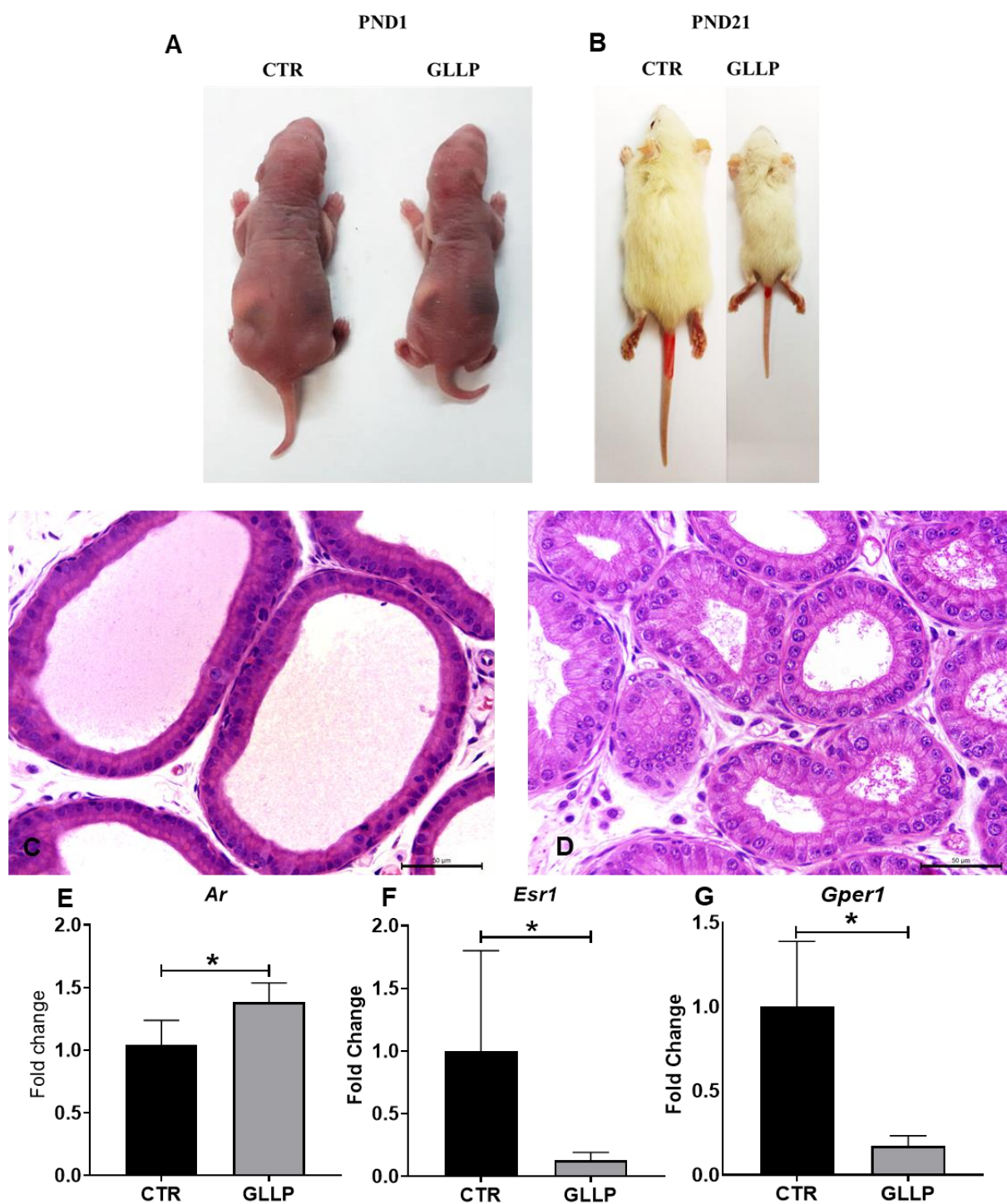


Figure 2. The representation of animals in the postnatal day (PND) 1 (A) and PND 21 (B) and Representative histological sections of ventral prostate (VP) lobes from the CTR (C) and GLLP (D) groups at PND 21 stained with hematoxylin-eosin (HE) for a general view of glandular morphology. Representative image of RT-qPCR analysis of hormones receptors in the ventral prostate (VP) from all experimental groups at PND 21 (E-G). Androgen receptor (Ar) (E). Estrogen receptor alpha (Esr1) (F). G Protein-Coupled Estrogen Receptor 1 (Gper1) (G). Data are expressed as mean $\pm$ SD. *Signifies a statistical difference between experimental groups with $p < 0.05$. Scale bar 50 μ m.

Table 2. Results of the ways in which miRNAs are secreted generated by the miRandola database

miRNA	Argonaute2	Circulating	Exosome	Microvesicle
miR122	X	X	X	X
miR133b	-	X	X	-
miR184	-	X	X	-
miR206	-	X	X	-
miR299-5p	-	X	-	-
miR33	-	-	X	-
miR342-5p	-	X	X	-
miR412	-	X	-	-
miR433	-	X	X	-
miR483-3p	-	X	X	-
miR483-5p	-	X	X	X
miR493-5p	-	X	-	-
miR99a-5p	-	-	X	-

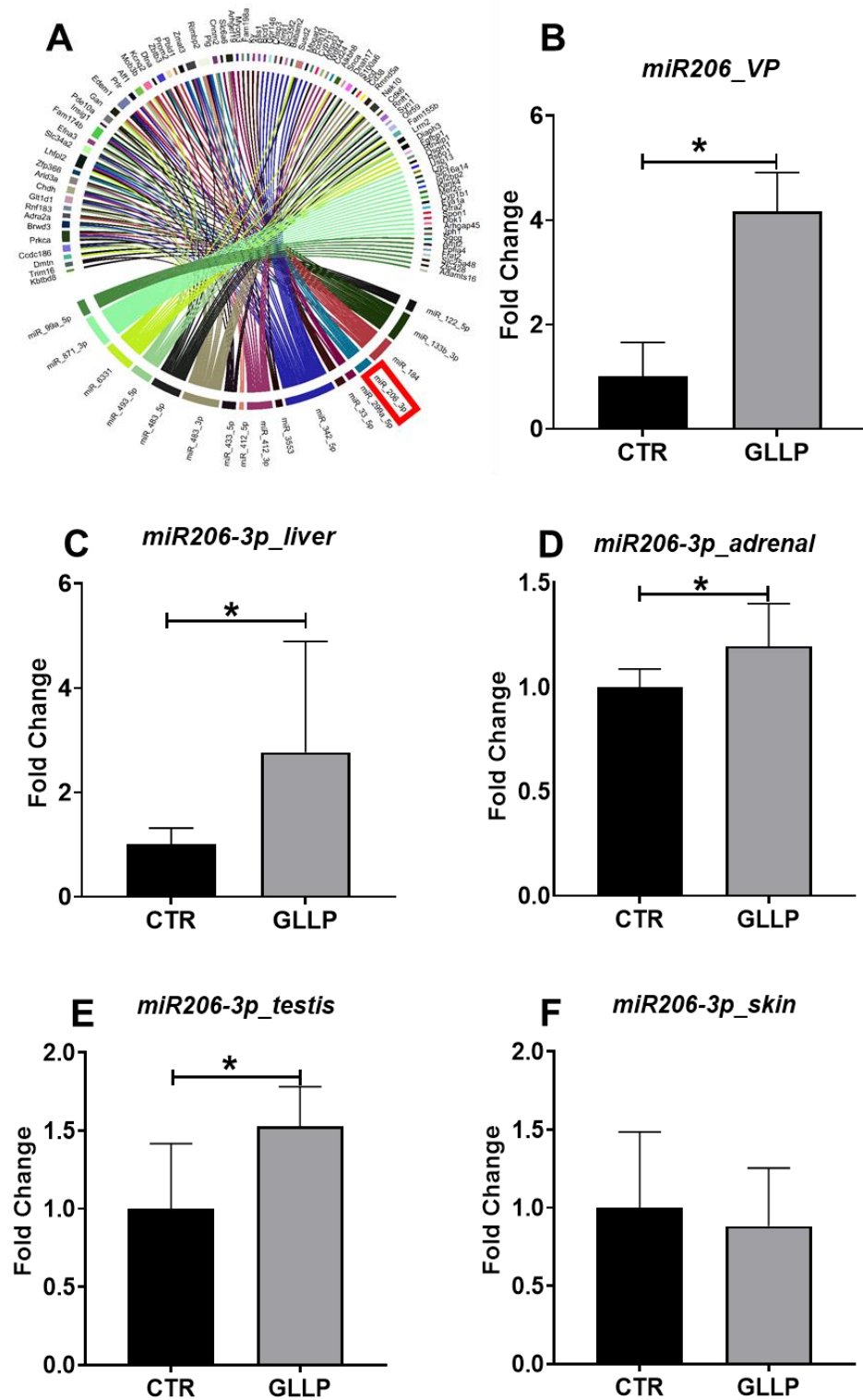
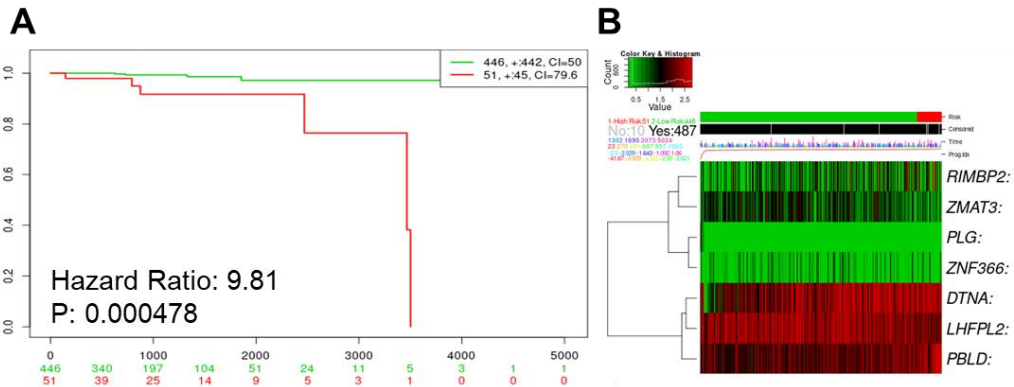


Figure 3. Circos plot graphic identifying the network of miRNAs-mRNA DE in the Ventral Prostate (VP) the animals of GLLP group (A). RT-qPCR analysis of ventral prostate (VP) (B), liver (C), adrenal (D), testis (E) and skin (F) from all experimental groups at PND 21, the results of relative expression of miR206-3p. Data are expressed as mean $\pm$ SD. *Signifies a statistical difference between experimental groups with $p < 0.05$.

Table 3. The main functions of the target miR206-3p genes that are differentially expressed in the VP of animals submitted to MLPD.

mRNA	Gene name	Functional	Ref
<i>Zmat3</i>	Zinc Finger Matrin-Type 3	Gene encoding of protein that acts in conjunction with p53 that acting on tumor suppression, inhibiting the growth of tumor cells, including skin and liver cancer. Its role is associated with the regulation of splicing in the p53 gene, and the presence of SNP leads to loss of cell cycle control and regulation of apoptotic processes.	[87–89]
<i>Plg</i>	Plasminogen	Encoding of a serine protease that circulates in the bloodstream and that when cleaved gives rise to an active serine protease, plasmin. Plasmin when cleaved gives rise to angiostatin that inhibits angiogenesis processes. Plasminogen still adheres to the cell surface through receptors and acts by degrading the extracellular matrix, mediating inflammation and tissue healing. In PCa, the role of enzymes that activate plasminogen is known, which degrade the extracellular matrix.	[90–93]
<i>Rimbp2</i>	RIMS Binding Protein 2	The protein encoded by this gene is associated with synaptic processes, activating the Ca ²⁺ channels and recruiting vesicles to the release sites, favoring synaptic transmission.	[94, 95]
<i>Dtna</i>	Dystrobrevin Alpha	Member of the family of dystrophin that make up the sarcolemma that surrounds muscle fibers, and is associated with neuromuscular junctions, the stability of acetylcholine receptors	[96, 97]
<i>Lhfp12</i>	LHFPL Tetraspan Subfamily Member 2	Subunit of transmembrane protein complexes, there are no reports as to its functional role in cell physiology, however studies show that it is expressed in both male and female reproductive tissues, and that mutations in this gene lead to decreased fertility in both sexes.	[98]
<i>Zfp366</i>	Zinc Finger Protein 366	A transcription corepressor, being associated with the estrogen alpha receptor (ESR1) and histone deacetylase activity. Its low expression is associated with delayed development of the mammary gland. In PCa, the gene and protein expression of ZFP366 is low when compared to normal tissues and the increase of its expression in tumor cells is associated with the repression of cell growth.	[99–101]
<i>Pbld1</i>	Phenazine Biosynthesis Like Protein Domain Containing 1	Encoded protein is linked to hepatocellular carcinogenesis processes, acting as a response to increased tissue oxidative stress, acting as a tumor suppressor and decreasing its expression is associated with a worse prognosis for patients with liver cancer.	[102, 103]



C

	ZMAT3	PLG	ZNF366	DTNA	LHFPL2	PBLD
Prostate	HPA027569 Male, age 37 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 3497	HPA021602 Male, age 51 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 2053	HPA023128 Male, age 51 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 2053	HPA071177 Male, age 37 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 3497	HPA042402 Male, age 51 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 2053	HPA038036 Male, age 48 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 2472
Glandular cells	HPA027569 Male, age 37 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 3497	HPA021602 Male, age 51 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 2053	HPA023128 Male, age 51 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 2053	HPA071177 Male, age 37 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 3497	HPA042402 Male, age 51 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 2053	HPA038036 Male, age 48 Prostate (T-77100) Normal tissue, NOS (M-00100) Patient id: 2472
Staining:	Low	Low	Low	Low	Medium	Low
Intensity:	Weak	Weak	Weak	Moderate	Moderate	Weak
Quantity:	75%-25%	>75%	75%-25%	<25%	>75%	75%-25%
Location:	Cytoplasmic/ membranous	Cytoplasmic/ membranous	Nuclear	Cytoplasmic/ membranous	Cytoplasmic/ nuclear	Cytoplasmic/ membranous
Prostate cancer	HPA027569 Male, age 81 Prostate (T-77100) Adenocarcinoma, Low grade (M-814031) Patient id: 4891	HPA021602 Male, age 50 Prostate (T-77100) Adenocarcinoma, High grade (M-814033) Patient id: 3559	HPA023128 Male, age 52 Prostate (T-77100) Adenocarcinoma, Low grade (M-814031) Patient id: 3558	HPA071177 Male, age 57 Prostate (T-77100) Adenocarcinoma, Low grade (M-814031) Patient id: 4333	HPA042402 Male, age 59 Prostate (T-77100) Adenocarcinoma, High grade (M-814033) Patient id: 3558	HPA038036 Male, age 83 Prostate (T-77100) Adenocarcinoma, High grade (M-814033) Normal tissue, NOS (M-00100) Patient id: 2502
Tumor cells	HPA027569 Male, age 81 Prostate (T-77100) Adenocarcinoma, Low grade (M-814031) Patient id: 4891	HPA021602 Male, age 50 Prostate (T-77100) Adenocarcinoma, High grade (M-814033) Patient id: 3559	HPA023128 Male, age 52 Prostate (T-77100) Adenocarcinoma, Low grade (M-814031) Patient id: 3558	HPA071177 Male, age 57 Prostate (T-77100) Adenocarcinoma, Low grade (M-814031) Patient id: 4333	HPA042402 Male, age 59 Prostate (T-77100) Adenocarcinoma, High grade (M-814033) Patient id: 3558	HPA038036 Male, age 83 Prostate (T-77100) Adenocarcinoma, High grade (M-814033) Normal tissue, NOS (M-00100) Patient id: 2502
Staining:	Not detected	Not detected	Not detected	Low	Low	Low
Intensity:	Negative	Negative	Weak	Moderate	Weak	Weak
Quantity:	None	None	<25%	<25%	75%-25%	>75%
Location:	None	None	Nuclear	Cytoplasmic/ membranous	Cytoplasmic/ membranous	Cytoplasmic/ membranous

Figure 4. Correlation of targets of miR206-3p (ZMAT3, PLG, ZNF366, DTNA, LHFPL2, and PBLD) with prognosis in patients of PCa data from SurvExpress. Overall survival curves comparing patients at high (red line) and low (green line) risks (A). Heatmap for each of the predicted miR206-3p targets in patients with high and low-risk PCa. The lines represent the genes, and the bars represent the expression in each of the patients, the green bars mean decreased expression and the red bars mean increased expression (B). Immunostaining of normal and human prostate tumor samples for six target genes of miR206-3p. The genes ZMAT3, PLG, ZNF366 and LHFPL2 showed downregulated in our samples and GEPIA database (<http://gepia.cancerpku.cn/>) using immunohistochemical data available at the Human Protein Atlas database (<https://proteintlas.org/>), DNTA and PBLD are downregulated in our samples and show did not differ in immunostaining between normal and tumor tissues (C).

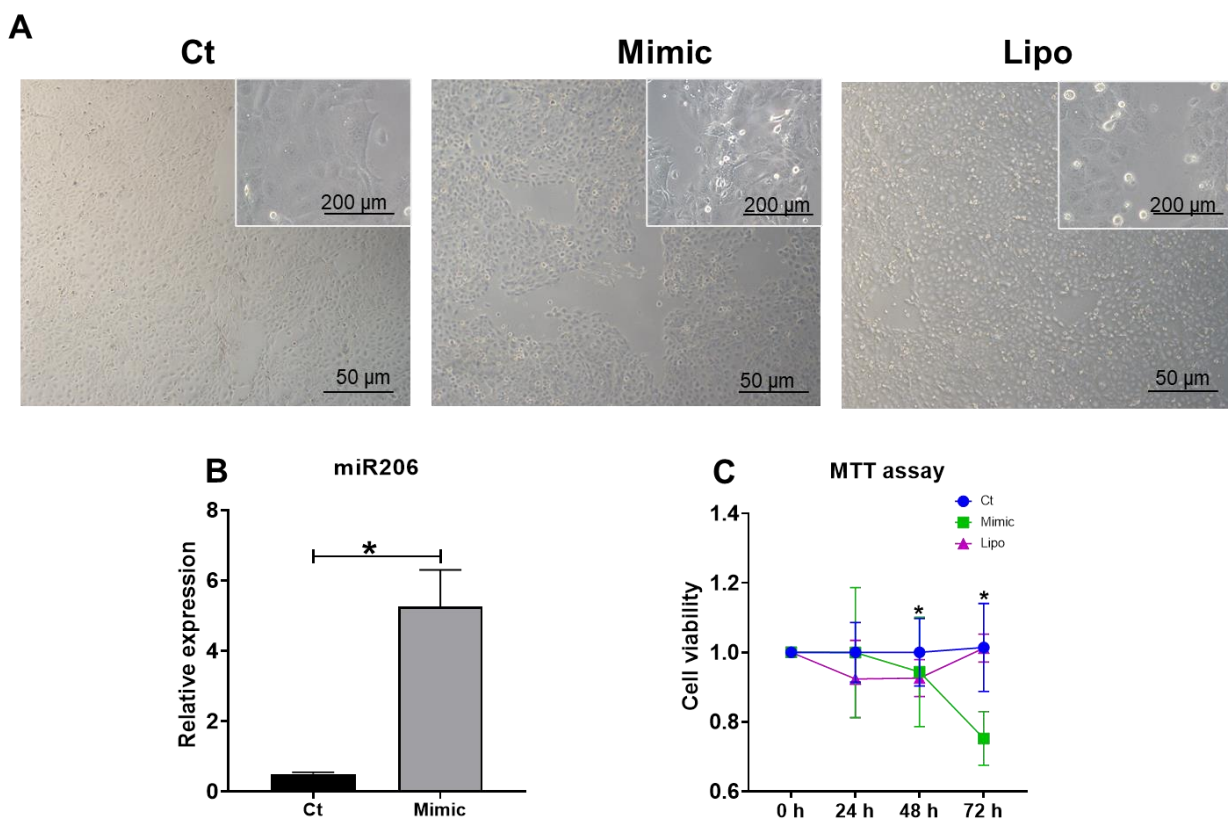


Figure 5. Representative image of assays performed in cell culture on benign prostatic epithelial cells (PNT2 lineage). (A) PNT2 lineage cells in different treatments: Ct, Mimic, and Lipo, after 24 hours of transfection with the miR-206-3p mimic. (B) miR-206-3p level in PNT2 control cells and transfected with miR-206-3p mimic after 24 hours. (C) Cell viability of cell lineage PNT2 in different treatments: Ct, Mimic, and Lipo after 24, 48, and 72 hours of transfection with the miR-206-3p mimic or not. Ct: control cells, mimic: cells subjected to miR206-3p mimic treatment. Lipo: cells treated with lipofectamine. Data are expressed as mean $\pm$ SD. *Signifies a statistical difference between experimental groups with $p < 0.05$. Figures with scale of 50 μ m, details with 200 μ m

Supplementary Material

Maternal malnutrition alters steroid biosynthesis and increase the expression of tumor suppressor miR206-3p in the ventral prostate of young rat offspring

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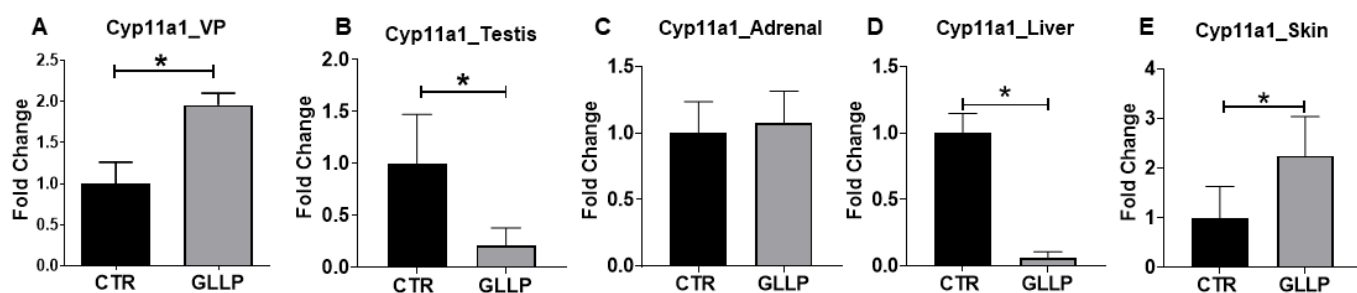
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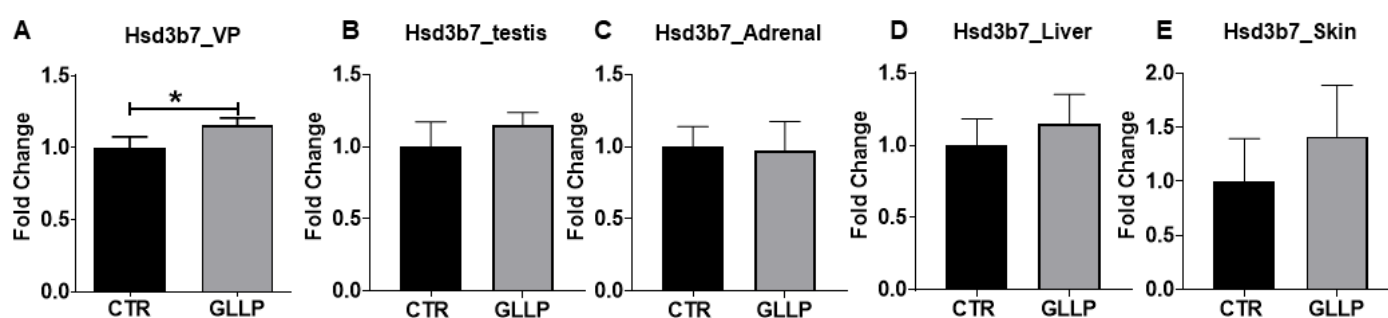
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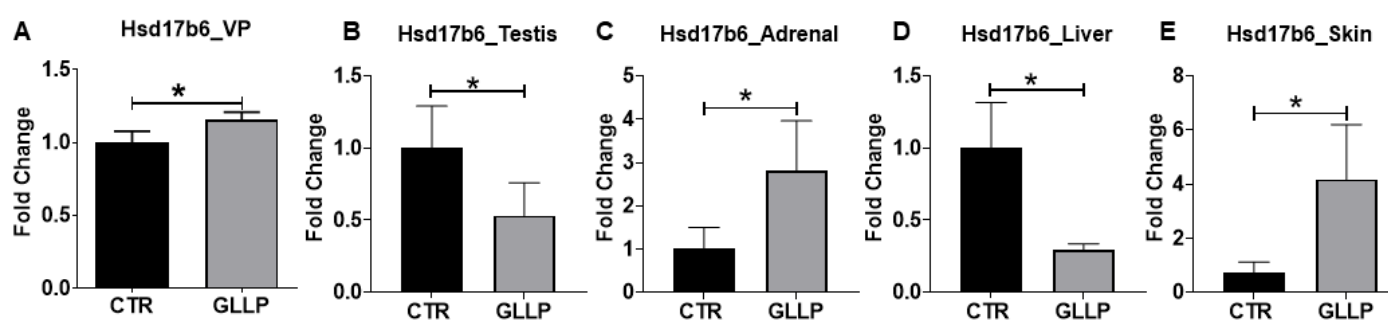
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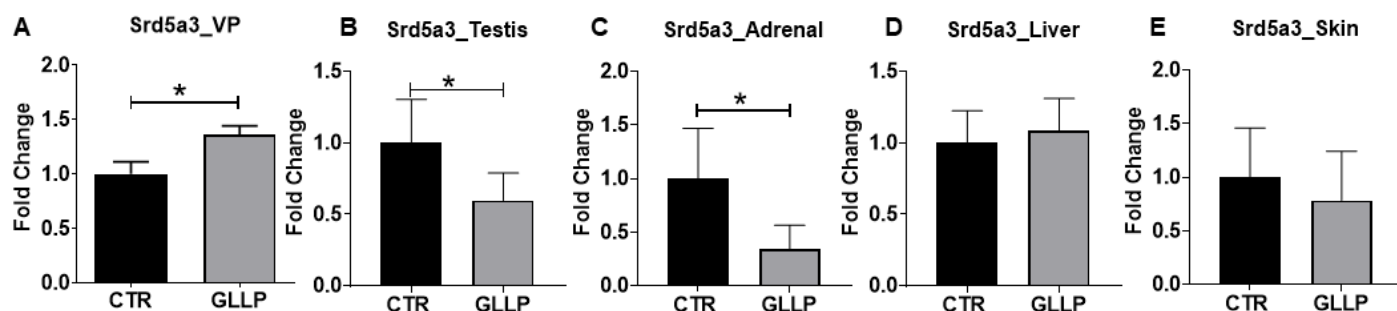
Supplementary Figure 1. Representative image of RT-qPCR analysis of Cyp11a1 gene, in all experimental groups on the animals postnatal day (PND21). A: Ventral Prostate. B: testis. C: adrenal. D: Liver. E: Skin. Data are expressed as mean $\pm$ SD. *Signifies a statistical difference between experimental groups with $p < 0.05$.



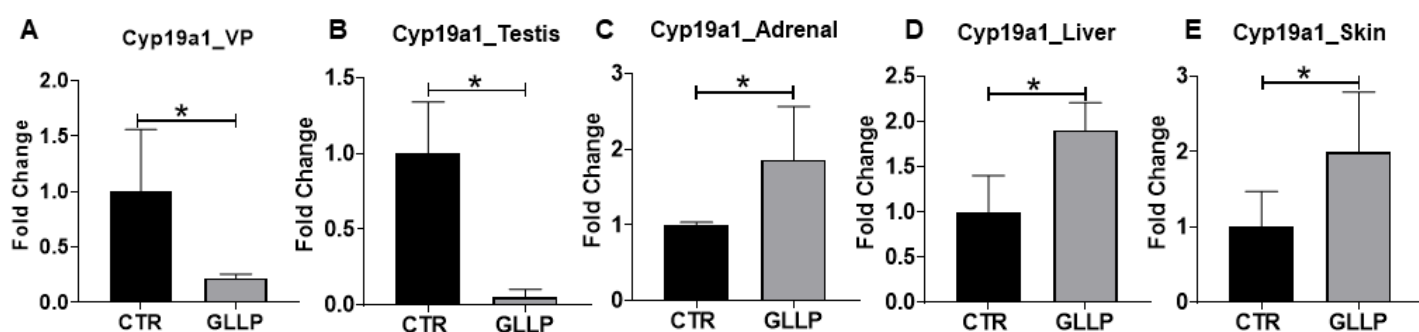
Supplementary Figure 2. Representative image of RT-qPCR analysis of Hsd3b7 gene, in all experimental groups on the animals postnatal day (PND21). A: Ventral Prostate. B: testis. C: adrenal. D: Liver. E: Skin. Data are expressed as mean $\pm$ SD. *Signifies a statistical difference between experimental groups with $p < 0.05$.



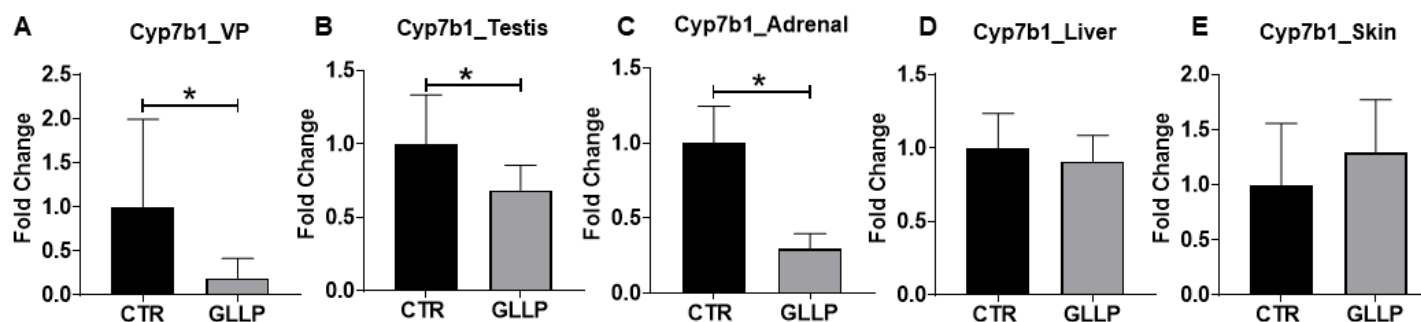
Supplementary Figure 3. Representative image of RT-qPCR analysis of Hsd17b6 gene, in all experimental groups on the animals postnatal day (PND21). A: Ventral Prostate. B: testis. C: adrenal. D: Liver. E: Skin. Data are expressed as mean $\pm$ SD. *Signifies a statistical difference between experimental groups with $p < 0.05$.



Supplementary Figure 4. Representative image of RT-qPCR analysis of Srd5a3 gene, in all experimental groups on the animals postnatal day (PND21). A: Ventral Prostate. B: testis. C: adrenal. D: Liver. E: Skin. Data are expressed as mean $\pm$ SD. *Signifies a statistical difference between experimental groups with $p < 0.05$.



Supplementary Figure 5. Representative image of RT-qPCR analysis of Cyp19a1 gene, in all experimental groups on the animals postnatal day (PND21). A: Ventral Prostate. B: testis. C: adrenal. D: Liver. E: Skin. Data are expressed as mean $\pm$ SD. *Signifies a statistical difference between experimental groups with $p < 0.05$.



Supplementary Figure 6. Representative image of RT-qPCR analysis of Cyp7b1 gene, in all experimental groups on the animals postnatal day (PND21). A: Ventral Prostate. B: testis. C: adrenal. D: Liver. E: Skin. Data are expressed as mean $\pm$ SD. *Signifies a statistical difference between experimental groups with $p < 0.05$.

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