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Análise da expressão de miRNAs em células humanas de próstata infectadas com o Zika vírus

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Dedico este trabalho à minha família,
Anjos de Deus em minha vida.

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“Science is light. Light is precious in a world so dark. Begin at the beginning. Make some light” (Ciência é luz. A luz é preciosa em um mundo tão sombrio. Comece do começo. Faça alguma luz). Adaptado de Kate DiCamillo, The tale of Despereaux.

RESUMO

O vírus Zika (ZIKV) é um vírus do gênero *Flavivirus*. Em humanos, o modo de transmissão mais comum é através da picada do mosquito *Aedes aegypti*. Entretanto, evidências recentes têm sugerido a transmissão sexual do ZIKV. Embora alguns estudos preconizem o testículo e a próstata como os principais órgãos que colaboram nessa forma de transmissão, pouco se sabe sobre quais tipos celulares destes tecidos são mais suscetíveis a esse vírus. Além disso, a infecção com linhagens distintas do ZIKV em modelos *in vitro* e *in vivo* tem demonstrado que a resposta do hospedeiro à infecção é cepa-dependente. Descobertas recentes indicam que esse vírus desregula o perfil de miRNAs celulares e que esse é um importante evento ao longo da infecção. Nesse estudo, avaliamos a suscetibilidade, a permissividade e o perfil de miRNAs celulares de células humanas epiteliais prostáticas (PNT1A) à infecção por duas diferentes cepas do ZIKV, uma cepa africana clássica, MR766 ($ZIKV^{MR766}$) e uma circulante no Brasil, $ZIKV^{BR}$. Para isso, células PNT1A foram infectadas com as cepas do ZIKV e submetidas ao ensaio de imunofluorescência indireta para a proteína do envelope viral; foram monitoradas quanto à produção de partículas virais infecciosas e cópias de RNA viral por ensaio de formação de placas e qPCR, respectivamente e analisou-se o perfil de miRNAs celulares por *PCR array*. Os resultados mostraram que células prostáticas humanas foram suscetíveis e permissivas à infecção pelo ZIKV e não apresentaram nenhuma imposição quanto a infecção por linhagens distintas desse vírus. As cepas testadas não diferiram quanto à cinética de replicação em células de próstata, mas diferiram quanto á modulação da expressão de miRNAs celulares. Após a infecção, 16 miRNAs celulares foram modulados em células de próstata, um pequeno grupo de 6 miRNAs foram modulados por ambas as cepas, enquanto um grupo de 10 miRNAs foram modulados exclusivamente pelo $ZIKV^{BR}$. Análises *in silico* identificaram que o grupo exclusivo de miRNAs regulados na infecção pela cepa brasileira do ZIKV pode regular genes e vias associadas a inflamação, imunidade, sobrevivência celular e proliferação celular. Nossos resultados indicam que a próstata pode ter um importante papel na transmissão sexual do ZIKV e destaca que cepas diferentes do vírus Zika podem induzir uma expressão diferencial de miRNAs celulares, a qual pode influenciar nas diferenças fisiopatológicas após infecção por diferentes cepas.

Palavras chaves: Próstata. Transmissão sexual. miRNAs. Cepa Brasileira do Zika vírus. Cepa MR766. Zika vírus.

ABSTRACT

Zika virus (ZIKV) is a virus from the *Flavivirus* genus. In humans this virus is transmitted mainly by *Aedes aegypti* mosquitoes. However, recent evidences indicate the occurrence of sexual transmission. Although some studies have indicated testes and prostate as the main organs that collaborate in this transmission, little is known about which cell types in these tissues are more susceptible to this virus. In addition, infection with distinct ZIKV strains in some models *in vitro* and *in vivo* has demonstrated that the host's response to infection is strain-dependent. Recent findings suggest that this virus deregulates host miRNA profile and that this is an important event throughout the course of the infection. Herein, we evaluated the susceptibility, the permissiveness and the cellular miRNA profile of human prostatic epithelial cells (PNT1A) to two different strains of ZIKV, a classical African strain, MR766 ($ZIKV^{MR766}$) and a Brazilian strain, $ZIKV^{BR}$. So, we infected PNT1A cells with ZIKV strains and performed an indirect immunofluorescence assay for protein envelope; monitored infectious viral particles production and RNA viral copies by plate assay and qPCR, respectively, and analyzed the miRNA cellular profile by PCR array. Our results demonstrated that human prostate cells are susceptible and permissive to ZIKV infection and did not present any imposition regarding infection by distinct strains of this virus. The strains did not differ in the kinetics of replication in prostate cells, but presented differences in miRNA's cell expression modulation. After infection, 16 miRNAs were modulated in prostate cells, a small group of 6 miRNAs were modulated by both strains while a set of 10 miRNAs showed to be modulated exclusively by $ZIKV^{BR}$. *In silico* analyses predicted that the miRNA upregulated exclusively by the infection by the Brazilian strain may regulate genes and pathways associated to inflammation, immunity, cell survival and cell proliferation. Taken together, our results indicate that prostate may be an important role in the sexual transmission of ZIKV and highlights that different strains of ZIKV may induce a differential host miRNA expression which may influence the differences in the physiopathology presented after the infection by different strains.

Keywords: Prostate. Sexual transmission. miRNAs. Zika virus Brazilian strain. MR766 strain. Zika virus.

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LISTA DE ABREVIATURAS E SIGLAS

µL	Microlitro, do inglês *microliter*
3' UTR	Região não traduzível 3', do inglês *3' Untranslated Region*
5' UTR	Região não traduzível 5', do inglês *5' Untranslated Region*
AGO2	Componente catalítico argonauta 2 de RISC, do inglês *argonaute RISC catalytic component 2*
AKT1	Quinase serina/treonina AKT 1, do inglês *AKT serine/threonine kinase 1*
ATM	Quinase serina/treonina ATM, do inglês *ATM serine/threonine kinase*
BCL-2	Regulador da apoptose BCL2, do inglês *BCL2 apoptosis regulator*
BSA	Albumina de soro bovino, do inglês *bovine serum albumin*
C	Proteína capsídeo, do inglês *Capsid protein*
CCL2	Ligante de quimiocina 2 motivo C-C, do inglês *C-C motif chemokine ligand 2*
CCND1	Ciclina D1, do inglês *Cyclin D1*
CCND2	Ciclina D2, do inglês *Cyclin D2*
CDK 4	Quinase dependente de ciclina 4, do inglês *Cyclin-dependent kinase 4*
CDK 6	Quinase dependente de ciclina 6, do inglês *Cyclin-dependent kinase 6*
cDNA	Ácido desoxirribonucleico complementar, do inglês C*omplementary Deoxyribonucleic Acid*
CFLAR	CASP8 e FADD como reguladores de apoptose, do inglês *CASP8 and FADD like apoptosis regulator*
CHEK2	Ponto de verificação quinase 2, do inglês *Checkpoint kinase 2*
CSF	Fluido cerebroespinal, do inglês *cerebrospinal fluid*
CXCL8	Ligante de quimiocina do motive C-X-C 8, do inglês *C-X-C Motif Chemokine Ligand 8*
DAPI	*4',6'-diamino-2-fenil-indol*
DENV	Vírus da dengue, do inglês *Dengue virus*
DGCR8	Subunidade complexa do microprocessador DGCR8, do inglês *DGCR8 microprocessor complex subunit*
DICER	Dicer, Ribonuclease III, do inglês *dicer, ribonuclease III*

DMEM	Meio Eagle modificado de dulbecco, do inglês *Dulbecco's modified eagle medium*
DNA	Àcido desoxirribonucleico, do inglês *deoxyribonucleic acid*
DROSHA	Ribonuclease III Drosha, do inglês *drosha ribonuclease III*
E	Proteína envelope, do inglês *envelope protein*
EXP5	Exportina 5, do inglês *Exportin 5*
FAS	Receptor de morte na superfície celular Fas, do inglês *Fas cell surface death receptor*
FASLG	Ligante Fas, do inglês *Fas ligand*
FBS	Soro fetal bovino, do inglês *fetal bovine serum*
G-CSF	Fator estimulante de colônia granulócita, do inglês *Granulocyte-colony stimulating factor*
GTP	Guanosina trifosfato, do inglês *Triphosphate guanosine*
HBV	Vírus da hepatite B, do inglês *Hepatitis B virus*
HCC	Carcinoma hepatocelular, do inglês *Hepatocellular carcinoma*
HCV	Vírus da hepatite C, *Hepatitis C Virus*
HIV	Vírus da imunodeficiência humana, do inglês *Human Immunodeficiency Virus*
hpi	Horas pós-infecção, do inglês *hours post infection*
hsa	*Homo sapiens*
IDO	Indoleamina 2,3-dioxigenase, do inglês *indoleamine 2,3-dioxigenase*
IFA	Ensaio de imunofluorescência, do inglês *immunofluorescence assay*
IgG	Imunoglobulina G, do inglês *Immunoglobulin G*
IL-1b	Interleucina 1 beta, do inglês *Interleukin 1 beta*
IL-6	Interleucina 6, do inglês *Interleukin 6*
INF ɣ	Interferon gama, do inglês *Interferon gamma*
JEV	Vírus da encefalite japonesa, do inglês *Japanese encephalitis virus*
L-15	Meio Leibovitz L-15, do inglês *Leibovitz L-15 medium*
mAB	Anticorpo monoclonal, do inglês *monoclonal antibody*
MAPK1	Proteína quinase ativada por mitógeno 1, do inglês *Mitogen-activated protein kinase 1*

MAPK8	Proteína quinase ativada por mitógeno 8, do inglês *mitogen-activated protein kinase 8*
MCL-1	Regulador de apoptose MCL1, membro da família BCL2, do inglês *MCL1 apoptosis regulator, BCL2 family member*
MDM	Macrófago derivado de monócito, do inglês *monocyte-derived macrophage*
MDM2	Proto-oncogene MDM2, do inglês *MDM2 proto-oncogene*
miRISC	Complexo de silenciamento induzido pelo miRNA maduro, do inglês *microRNA-Induced Silencing Complex*
miRNAs	microRNAs
mL	Mililitro, do inglês *mililiter*
mRNA	Ácido ribonucléico mensageiro, do inglês *Messenger Ribonucleic Acid*
NF-kappa B	Fator nuclear kappa B, do inglês N*uclear Fator kappa B*
NS1	Proteína não estrutural 1, do inglês *non-structural protein 1*
NS2a	Proteína não estrutural 2 a, do inglês *non-structural protein 2 a*
NS2b	Proteína não estrutural 2b, do inglês *non-structural protein 2 b*
NS3	Proteína não estrutural 3, do inglês *non-structural protein 3*
NS4a	Proteína não estrutural 4 a, do inglês *non-structural protein 4 a*
NS4b	Proteína não estrutural 4 b, do inglês *non-structural protein 4 b*
NS5	Proteína não estrutural 5, do inglês *non-structural protein 5*
OMS	Organização mundial da saúde
OPAS	Organização Pan Americana da Saúde
P/S	Penicilina/estreptomicina, do inglês *penicillin-streptomycin*
PBS	Tampão fosfato salino, do inglês *phosphate-buffered saline*
PCR	Reação em cadeia da polimerase, do inglês *Polymerase Chain Reaction*
PFU/mL	Unidades formadoras de placas por mililitro, do inglês *Plaque forming unit per mililiter*
PI3K	Fosfatidilinositol 3-quinase, do inglês *Phosphatidylinositol 3-kinase*
PI3KCD	Quinase da subunidade catalítica delta da fosfatidilinositol 3-quinase, do inglês *Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta*

PKA	Proteína quinase 4, do inglês *protein kinase A*
pRb	Proteína retinoblastoma, do inglês *Retinoblastoma protein*
Pre-miRNA	miRNA precursor, do inglês *miRNA precursor*
Pri-miRNA	miRNA primário, do inglês *primary miRNA*
prM/M	Proteína pré-membrana/Membrana, do inglês *pre membrane protein/Membrane*
PTGS2	Endoperóxido de prostaglandina sintetase 2, do inglês *Prostaglandin-endoperoxide synthase 2*
qPCR	Reação em cadeia da polymerase quantitativa em tempo real, do inglês *Real Time Quantitative Polymerase Chain Reaction*
Ran-GTP	Proteína nuclear Ran de ligação a GTP, do inglês *Ran GTP-binding protein*
RISC	Complexo de silenciamento induzido por RNA, do inglês *RNA-induced silencing complex*
RLC	Complexo de carregamento de RISC, do inglês, *RISC-loading complex*
RNA	Ácido ribonucleico, do inglês *Ribonucleic acid*
ROS	Espécies reativas de oxigênio, do inglês *Reactive oxygen species*
RT	Temperatura ambiente, do inglês *Room temperature*
SD	Desvio padrão, do inglês *standart deviation*
SGB / GBS	Síndrome de Guillain-Barré do inglês *Guillain-Barre Syndrome*
STAT3	Transductor de sinal e ativador de transcrição 3, do inglês *Signal Transducer and Activator Of Transcription 3*
TARBP	Subunidade TARBP do complexo de carregamento RISC, do inglês *TARBP subunit of RISC loading complex*
TNF	Fator de necrose tumoral, do inglês *Tumor Necrosis Factor*
TNFRSF1A	Membro 1a da super família de receptores TNF, do inglês *TNF Receptor Superfamily member 1A*
TNFSF10	Membro 10 da super família TNF, do inglês *TNF superfamily member 10*
TNF-α	Fator de necrose tumoral alfa, do inglês *Tumor Necrosis factor alpha*
TP53	Proteína de tumor p53, do inglês *Tumor protein p53*

TRAILR	Receptor de ligante indutor de apoptose relacionado a TNF, do inglês *TNF-Related Apoptosis Inducing Ligand Receptor*
WNV	Vírus do oeste do Nilo, do inglês *West Nile virus*
YFV	Vírus da febre amarela, do inglês *Yellow fever virus*
ZIKV	Vírus da Zika, do inglês *Zika virus*
$ZIKV^{AF}$	Cepas africanas do vírus Zika
$ZIKV^{AS}$	Cepas asiáticas do vírus Zika
$ZIKV^{BR}$	Cepa Brasileira do vírus Zika, do inglês *Brazilian strain of Zika virus*
$ZIKV^{MR766}$	Cepa MR766 do vírus Zika, do inglês *MR766 strain of Zika virus*

Sumário

1. INTRODUÇÃO

A emergência do vírus Zika (ZIKV) nos últimos anos resultou em um quadro de infecções sem precedente em países da América Latina e Caribe, culminando com a sua declaração como quadro de emergência em saúde pública global de fevereiro a novembro de 2016 pela Organização Mundial da Saúde (OMS) (ORGANIZATION, 2016). Em resposta à declaração da OMS cientistas de diversos países começaram a investigar as propriedades do ZIKV e analisaram os casos recentes de microcefalia e Síndrome de Guillain-Barré (SGB). Com base em diversos desses estudos a OMS concluiu que no surto recente o ZIKV é o agente etiológico da Síndrome congênita do ZIKV e provável desencadeador da SGB (ORGANIZATION, 2016).

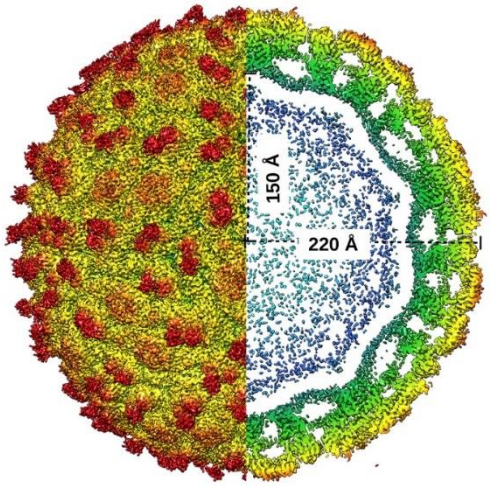
Apesar da relação entre o ZIKV e doenças neurológicas só ter sido proposta recentemente, o ZIKV foi descrito em 1947, sendo isolado a partir do sangue de um macaco-rhesus sentinela (*Macaca mulatta*), na floresta Zika, Uganda (DICK;KITCHEN; HADDOW, 1952). Os primeiros relatos de infecção humana por esse vírus ocorreram nesse mesmo país e na Tanzânia em 1952 (DICK;KITCHEN; HADDOW, 1952). Nos anos que se seguiram até 2006 os relatos de infecção humana por ZIKV foram esporádicos e restritos à países da África e sudeste asiático (PASSI *et al.*, 2017).

A primeira epidemia causada pelo ZIKV registrada fora das regiões africanas e asiáticas aconteceu na ilha de Yap, Micronésia, em 2007, onde estima-se que cerca de 73% da população foi infectada por esse vírus (DUFFY *et al.*, 2009). Posteriormente um segundo grande surto do ZIKV aconteceu entre 2013 e 2014 na Polinésia Francesa e foi acompanhado de um aumento expressivo de casos de SGB (CAO-LORMEAU et al., 2016). Atualmente, a infecção por ZIKV encontra-se emergente no hemisfério ocidental, possuindo casos relatados em vários países (CAO-LORMEAU *et al.*, 2016; LAZEAR; DIAMOND, 2016; PAN AMERICAN HEALTH, 2018). Um desses países é o Brasil, cuja infecção durante o período gestacional tem sido associada a Síndrome congênita do ZIKV em neonatos (FERNANDES *et al.*, 2016; YUN; LEE, 2017; FRANCA *et al.*, 2018).

O ZIKV é membro do gênero *Flavivirus* e junto com outros 2 gêneros constitui a família *Flaviviridae* (YE *et al.*, 2016). Assim como outros *Flavivirus*, o ZIKV é constituído por um genoma de RNA de fita simples de polaridade positiva (YE *et al.*, 2016). Apresenta-se como um vírion envelopado, icosaédrico de 40 a 50 nm de diâmetro (Figura 1) (SEVVANA *et al.*, 2018).

Seu genoma de aproximadamente 11.000 bases é traduzido na célula hospedeira em uma poliproteína viral, a qual é posteriormente processada dando origem a três proteínas estruturais: envelope (E), pré membrana/membrana (prM/M) e capsídeo (C); e, pelo menos, sete proteínas não estruturais (NS): NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5 (Figura 2) (WHITE *et al.*, 2016).

Figura 1: Reconstrução estrutural do vírion do ZIKV por microscopia eletrônica criogênica. A porção esquerda da figura refere-se ao envelope viral, enquanto do lado direito estão representadas estruturas internas, como o nucleocapsídeo.



Fonte: (SEVVANA *et al.*, 2018)

Figura 2: Representação esquemática do genoma do vírus Zika. As proteínas estruturais, C, prM/M e E são demonstradas em preto, enquanto as proteínas não estruturais NS1, NS2a, NS2b, NS3, NS4a, NS4b e NS5 estão representadas em azul. Flanqueando cada extremidade do genoma estão ilustradas as regiões não traduzíveis 5' e 3' (5'-UTR e 3'-UTR).

Fonte: modificado a partir de Ye *et al*., 2016.

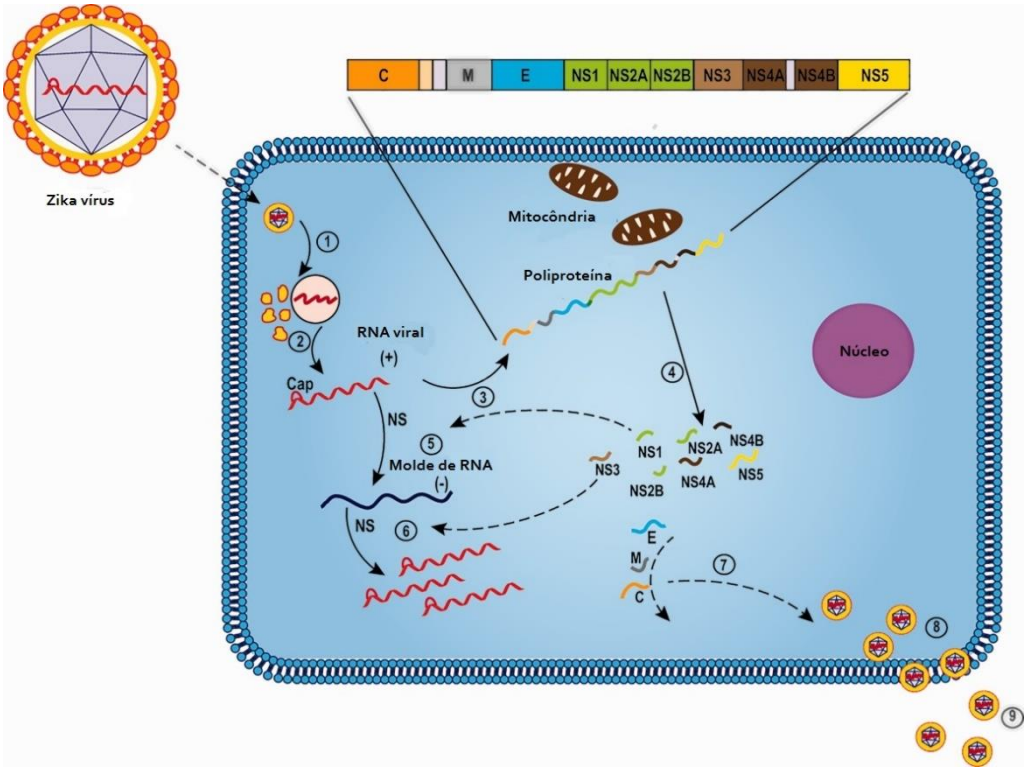
As proteínas estruturais E e M são essenciais para a infecção viral, participando do reconhecimento da célula hospedeira, ligação ao receptor de superfície celular e da endocitose mediada por receptores para a penetração viral (KAUFMANN; ROSSMANN, 2011; PERERA-LECOIN *et al.*, 2013). Já as proteínas NS atuam principalmente na replicação do ZIKV, e algumas funções ainda precisam ser decifradas. Apesar de ainda não ser totalmente conhecida a função da proteína NS1, as evidências disponíveis indicam que ela apresenta um papel inicial na replicação de *Flavivirus*, podendo atuar na formação do complexo de replicação (LINDENBACH; RICE, 1999; AKEY *et al.*, 2015). A proteína NS2a também participa no processo de replicação viral, e especula-se que possa estar associada com a encapsulação do genoma viral (KUMMERER; RICE, 2002; LEUNG *et al.*, 2008). Já as proteínas NS2b e NS4a são co-fatores para a atividade da NS3, modulando sua função de helicase (MURRAY;JONES; RICE, 2008).

A proteína NS3 é uma enzima multifuncional, pois é uma RNA- helicase além de apresentar uma função proteolítica no processamento da proteína estrutural C (MURRAY;JONES; RICE, 2008). A proteína NS4b, assim como a NS4a, é uma proteína hidrofóbica, e acredita-se que seja um componente da replicase (complexo de replicação viral) (CHAMBERS *et al.*, 1990). Já a proteína NS5 apresenta papel central na replicação de *Flavivirus*, pois possui atividades de RNA polimerase dependente de RNA e de metiltransferase, essenciais para a síntese de RNA viral e adição de CAP no genoma viral (MURRAY;JONES; RICE, 2008).

O processo de infecção viral se inicia com a adsorção do ZIKV à uma célula suscetível pela interação de glicoproteínas da superfície viral com receptores da superfície celular, culminando

com a internalização do vírus em vesículas revestidas por clatrina, por meio de endocitose mediada por receptores. Posteriormente, uma mudança no pH interno endossomal resulta em mudanças conformacionais no vírion e liberação do genoma viral no citoplasma da célula hospedeira. O genoma de polaridade positiva do ZIKV é então traduzido em uma poliproteína precursora por meio de enzimas virais e celulares. Modificações co-traducionais e pós-traducionais originam as 3 proteínas virais estruturais e 7 proteínas não-estruturais. A replicação do genoma do ZIKV ocorre em vesículas, iniciando-se pela síntese de intermediários de replicação (RNA fita simples de polaridade negativa), que será utilizado como molde para a síntese de novos genomas de polaridade positiva, que deverá compor novos vírions. Após a síntese de todos os componentes virais, a montagem dos novos vírions ocorre na superfície do retículo endoplasmático, seguida por brotamento. Seguindo a rota secretora da célula hospedeira, os novos vírions dirigem-se à rede trans-Golgi, onde sofrem maturação e posteriormente são liberados da célula hospedeira por exocitose (Figura 3) (WHITE *et al.*, 2016).

Figura 3: Representação esquemática do ciclo de vida do ZIKV.



Fonte: modificado a partir de (White *et al.*, 2016)

A forma mais comum de transmissão do ZIKV ocorre por meio da picada de mosquitos do gênero *Aedes* (AGUMADU; RAMPHUL, 2018). Recentemente novas evidências sugerem novas vias de transmissão do ZIKV: perinatal (BESNARD *et al.*, 2014), contato sexual entre homem e mulher (RUSSELL *et al.*, 2017), entre homens (DECKARD *et al.*, 2016) e transfusão de sangue (MUSSO *et al.*, 2014; MARANO *et al.*, 2016). Estas vias de transmissão elevam o risco de epidemias em territórios que não apresentam a presença do vetor artrópode. A maioria das pessoas infectadas com ZIKV não apresentam sintoma algum, mas esses quando presentes assemelham-se aos de outras arboviroses filogeneticamente próximas, sendo os mais comuns febre, dores musculares, erupções cutâneas, dores de cabeça, vermelhidão nos olhos e dores articulares (DIAS *et al.*, 2018).

A partir de amostras isoladas das infecções na África e Ásia por ZIKV foram identificadas 3 linhagens geneticamente e geograficamente distintas desse vírus: do leste africano, do oeste africano e asiática (FAYE *et al.*, 2014). A recente emergência do ZIKV nas Américas resultou em novas análises filogenéticas, as quais revelaram que as cepas circulantes na América Latina

derivaram-se das cepas asiáticas e constituem dentro dessa linhagem, um novo clado americano (YE *et al.*, 2016).

A epidemia de infecções pelo ZIKV tem se espalhado rapidamente pelo hemisfério ocidental. A Organização Pan Americana da Saúde (OPAS) registrou 223.477 casos autóctones de ZIKV , dos quais 61% ocorreram no Brasil no período entre 2015 e janeiro de 2018 (PAN AMERICAN HEALTH, 2018). Neste mesmo período também foram registrados 3.720 casos da síndrome congênita associada com a infecção pelo ZIKV, dos quais 79% acometeram fetos brasileiros, enquanto que os casos restantes aconteceram em países da América do Norte, América Latina, Caribe e Caribe não latino (PAN AMERICAN HEALTH, 2018).

Estudos com o ZIKV tem relatado um amplo tropismo tecidual e celular desse vírus (CHAN *et al.*, 2016; MINER; DIAMOND, 2017). O RNA viral tem sido isolado a partir de tecido cerebral fetal (MLAKAR *et al.*, 2016), placenta (BHATNAGAR *et al.*, 2017; REAGAN-STEINER *et al.*, 2017), tecido ocular, útero, vagina, testículo e fluídos corporais humanos (MINER; DIAMOND, 2017). A presença do vírus nesses tecidos tem estimulado estudos que ajudam a explicar a relação entre a infecção por ZIKV e microcefalia (GARCEZ *et al.*, 2016), onde foram sugeridos potenciais rotas de transmissão perinatal (TABATA *et al.*, 2016) e alertado para a persistência desse vírus nos sistemas reprodutores (BARZON *et al.*, 2016; MOREIRA *et al.*, 2017).

Além da grande preocupação da relação do ZIKV com o sistema neural, a sua relação com o sistema reprodutor humano também tem sido alvo de diversos grupos de pesquisas que estão estudando o potencial do vírus em se replicar no sistema urogenital masculino. Essa problemática surgiu a partir do isolamento do RNA viral a partir de urina e sêmen de homens infectados pelo ZIKV (PAZ-BAILEY *et al.*, 2018). Adicionalmente, o RNA desse vírus também tem sido encontrado no sêmen de homens assintomáticos (MUSSO *et al.*, 2017) e sintomáticos (MANSUY *et al.*, 2016; TURMEL *et al.*, 2016; JOGUET *et al.*, 2017) para a febre do Zika. Essa persistência e presença indica que tecidos urogenitais humanos são potenciais reservatórios do ZIKV.

Até o momento os principais órgãos do sistema reprodutor masculino inferidos como potenciais reservatórios e repositores do ZIKV são os testículos e a próstata (STASSEN *et al.*, 2018). A infecção por ZIKV levou a danos testiculares resultando em risco de infertilidade em ratos (GOVERO *et al.*, 2016; MA *et al.*, 2016) e um estudo *in vitro* demonstrou que esse vírus infecta células de Sertoli, sendo esse um importante passo para estabelecer a infecção persistente nesse órgão (SIEMANN *et al.*, 2017). Além disso foi relatado que células prostáticas estromais

humanas são suscetíveis e permissivas ao ZIKV, produzindo partículas infecciosas de forma significante (CHAN *et al.*, 2016; SPENCER *et al.*, 2018).

Estudos clínicos relataram a persistência do RNA do ZIKV por um pouco mais do que um mês na urina e até 370 dias no sêmen de pacientes infectados (BARZON *et al.*, 2016; BARZON *et al.*, 2018). Outro estudo com homens vasectomizados infectados com ZIKV revelaram a presença desse vírus em amostras de sêmen, apoiando a hipótese de que um órgão mais distal, como a próstata, é também um possível sítio de replicação para esse vírus no sistema reprodutor humano (ARSUAGA *et al.*, 2016; FROESCHL *et al.*, 2017).

Não obstante todas as questões que ainda precisam ser respondidas sobre o ZIKV e sua relação com o sistema reprodutor, alguns estudos tem observado que diferentes cepas do ZIKV podem apresentar diferentes padrões de infectividade e induzir respostas celulares diferentes. Demonstrou-se que cepas africanas do ZIKV ($ZIKV^{AF}$) apresentam maior infectividade e replicação em células-tronco neurais do que uma cepa asiática ($ZIKV^{AS}$). As cepas africanas induziram uma maior taxa de morte celular e resposta antiviral em comparação com a cepa $ZIKV^{AS}$ (SIMONIN *et al.*, 2016). Já um estudo em ratos observou que cepas $ZIKV^{AF}$ induzem danos mais graves à estrutura testicular do que cepas $ZIKV^{AS}$ (GOVERO *et al.*, 2016). Esses resultados sugerem que diferentes cepas podem conduzir a diferenças fisiopatológicas e moleculares durante a infecção por ZIKV.

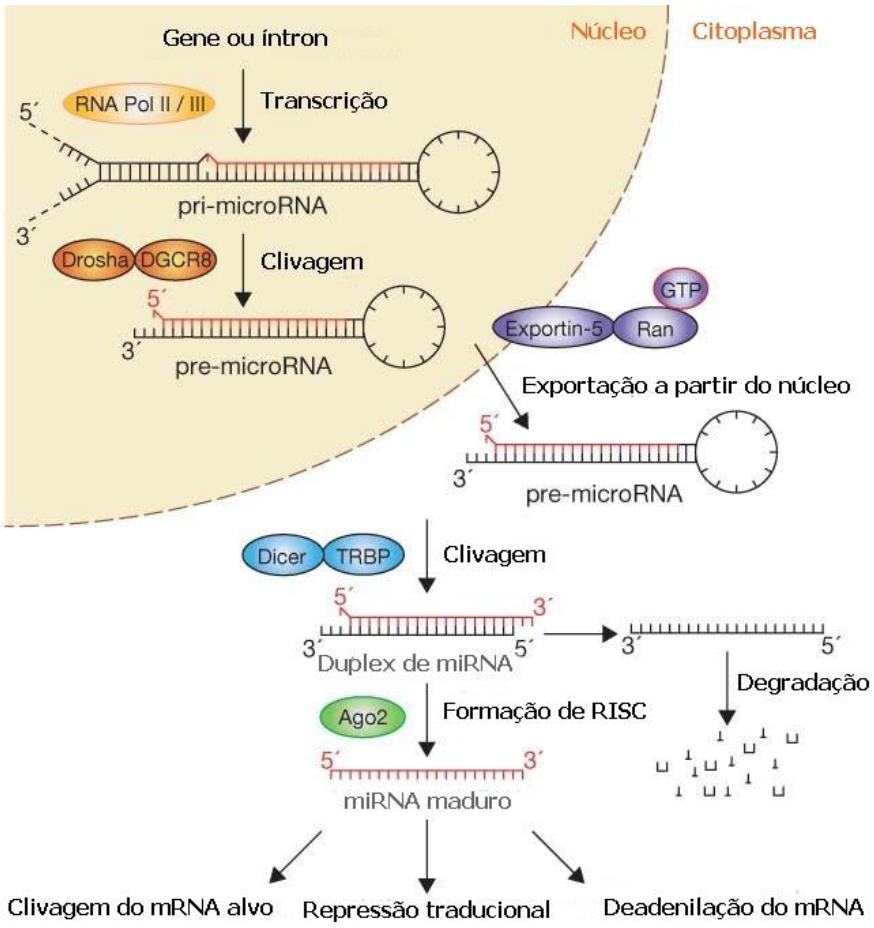
Entre as moléculas que podem ser analisadas para evidenciar diferença na resposta celular estão os microRNAs (miRNAs), pequenos RNAs não codificantes que regulam pós-transcricionalmente a expressão gênica e cujas funções estão associadas à vários processos celulares (BARTEL, 2004). Os miRNAs podem se originar por diversas vias, sendo a mais comum a via canônica, a qual envolve a transcrição do miRNA a partir do gene codificante, sua maturação e incorporação no complexo de silenciamento induzido por RNA (RISC, do inglês *RNA-induced silencing complex*) (KIM;HAN; SIOMI, 2009).

O *loci* genômicos de miRNAs são variáveis, de forma geral encontram-se isolados ou agrupados em regiões intergênicas ou em íntrons e éxons de genes codificantes (PEREIRA, 2015). A transcrição desses genes é feita pela RNA polimerase II, originando uma molécula com estrutura de "*stem-loop*" denominada miRNA primário (pri-miRNA). Posteriormente essa molécula é processada pela enzima Drosha juntamente com a proteína DGCR8 (*DGCR8 microprocessor complex subunit*), as quais clivam o pri-miRNA em um miRNA precursor (pre-miRNA), em forma

de “*hairpin*”. Essas etapas iniciais ocorrem no núcleo celular e a próxima etapa do processamento envolve a exportação do pre-miRNA para o citoplasma. Esse transporte é feito por um complexo constituído pela proteína Exportina 5 (Exp5) e pela proteína nuclear Ran de ligação a GTP (Ran-GTP) e ocorre através de poros nucleares (PEREIRA, 2015).

Uma vez no citoplasma, o pre-miRNA é liberado do complexo após hidrolisação de GTP, e é posteriormente clivado pela enzima Dicer, originando um duplex de miRNA. A formação do complexo RISC ocorre em sequência, e envolve a ação de diversas proteínas. Em células humanas, Dicer que porta o duplex de miRNA é recrutada junto com a proteína Argonauta 2 (AGO2) pela proteína TARBP (*TARBP subunit of RISC loading complex*), formando o complexo RLC - complexo de carregamento de RISC (do inglês, *RISC-loading complex*). RLC atua transferindo o duplex de miRNA de Dicer para AGO2, formando miRISC. Há ainda certa controvérsia em como ocorre a escolha da fita guia que constituirá RISC, mas acredita-se que a fita do duplex de miRNA que apresenta uma relativa instabilidade na porção 5’ é selecionada como fita guia, enquanto a outra fita, a fita passageira, é degradada. Entretanto, ambos os membros do duplex podem se associar a RISC e serem funcionais. Assim RISC constitui-se por uma proteína AGO (1-4) e a fita guia de miRNA, figura 4 (PEREIRA, 2015).

Figura 4: Representação esquemática da via canônica de biogênese de miRNA.



Fonte: modificado a partir de (WINTER *et al.*, 2009).

Diante da ampla gama de moléculas precursoras até se alcançar o miRNA maduro, convencionou-se uma nomenclatura própria para a designação de cada estrutura. Os *loci* genômicos codificantes de miRNAs são abreviados pelo prefixo *MIR*, em itálico, enquanto que os pre-miRNAs recebem o mesmo prefixo, exceto pela grafia em itálico. Já as formas maduras são designadas pelo prefixo miR. Ainda, um prefixo de três letras precedendo o termo miR, refere-se a espécie, desse modo, "hsa-miR" refere-se a forma madura de miRNAs em *Homo sapiens.* Outra convenção foi adotada após se observar que ambas as fitas do duplex podem se ligar a RISC e exercer sua função. Desse modo, o sufixo "-5p" ou "-3p" indica a extremidade do pre-miRNA da qual se originou a forma madura. Assim, o miRNA hsa-miR-142-3p é originado a partir da extremidade 3' da molécula precursora, enquanto o hsa-miR-142-5p origina-se da extremidade 5' (PEREIRA, 2015).

Os miRNAs regulam pós-transcricionalmente a expressão gênica. Sua regulação pós-transcricional ocorre principalmente por meio da ligação de sequências complementares dos miRNAs, denominadas *seed* à região 3'UTR do RNA mensageiro (mRNA) (BARTEL, 2004). A regulação dos miRNAs pode ocorrer por diversos mecanismos, como: inibição da tradução (PILLAI *et al.*, 2005; CHENDRIMADA *et al.*, 2007), desestabilização do transcrito-alvo (LLAVE *et al.*, 2002; BEHM-ANSMANT *et al.*, 2006), silenciamento transcricional (WU *et al.*, 2010; YOUNGER; COREY, 2011), promoção da transcrição (ZHANG;FAN;GENG*; et al.*, 2014; ZHANG;FAN;ZHANG*; et al.*, 2014) e intensificação da tradução (VASUDEVAN;TONG; STEITZ, 2007; OROM;NIELSEN; LUND, 2008). Por esses mecanismos os miRNAs exercem papéis regulatórios em diversos processos biológicos, como organogênese, proliferação e diferenciação celular, apoptose, neurogênese, resposta imunológica e tumorigênese (BARTEL, 2004), tornando-se assim, uma importante molécula para estudo.

Além das suas funções regulatórias os miRNAs também estão envolvidos na infecção viral e na modulação da resposta da célula hospedeira (BAVIA *et al.*, 2016). Estudos têm demonstrado que durante a infecção com *Flavivirus* as células hospedeiras podem alterar sua expressão de miRNAs envolvidos em funções fisiológicas e estabelecer um quadro antiviral (BAVIA *et al.*, 2016). A alteração no perfil celular de miRNAs humanos em resposta à infecção viral pode ser resultado de duas diferentes tentativas: uma atuação da resposta imune inata da célula a fim de inibir a replicação do vírus ou uma estratégia viral a fim de induzir um ambiente intracelular mais favorável à sua replicação (UMBACH; CULLEN, 2009).

Estudos que visam correlacionar o perfil de miRNAs celulares com a infecção por *Flavivirus* tem sido amplamente relatados. A infecção com *West Nile virus* mostrou-se capaz de induzir a expressão do miR-532-5p em células renais, sendo um importante regulador da atividade antiviral, inibindo a replicação desse vírus (SLONCHAK *et al.*, 2015). De forma similar, o miR-30e apresentou atividade antiviral em células cervicais (HeLa) e pleurais (U937), inibindo a replicação do vírus da dengue, DENV (ZHU *et al.*, 2014). Em células hepáticas (Huh7) o DENV-2 alterou a expressão de 9 miRNAs em diferentes tempos pós infecção, alguns dos quais estão envolvidos com a resposta antiviral da célula (ESCALERA-CUETO *et al.*, 2015). De uma forma geral a infecção por *Flavivirus* induz a um aumento na expressão de grupos diversos de miRNAs, resposta associada a ativação de atividades antivirais das células hospedeiras.

A infecção por ZIKV alterou o perfil de expressão de miRNAs em astrócitos humanos. A infecção resultou na modulação de miRNAs que participam de processos relacionados ao ciclo de vida viral, ciclo celular, transcrição viral, resposta imune inata e processos virais (KOZAK *et al.*, 2017). Além disso a infecção por ZIKV em células de fígado, pulmão e rim alterou a expressão de genes codificadores de proteínas chaves na via dos miRNAs, como AGO2 que participa da formação do complexo de indução de silenciamento, além de DICER e DROSHA, que são proteínas chaves no processo de maturação dos miRNAs (FERREIRA *et al.*, 2018). Entretanto, desconhecem-se quais miRNAs possuem sua expressão alterada devido à infecção pelo ZIKV em células prostáticas e se a infecção por cepas de linhagens distintas desse vírus modula diferencialmente o perfil de miRNAs celulares.

2. OBJETIVOS

O presente trabalho teve como objetivos analisar *in vitro* a suscetibilidade, permissividade e o perfil de miRNAs celulares de células humanas epiteliais de próstata (células PNT1A) à infecção pelas cepas africana, $ZIKV^{MR766}$ e brasileira, $ZIKV^{BR}$ do vírus Zika e analisar, *in silico*, os genes alvos destes miRNAs e as vias nas quais eles estão envolvidos.

3. ARTIGO CIENTÍFICO

Title: Identification of miRNAs differentially expressed in human prostate cells infected by Zika virus.

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Abstract

Zika virus (ZIKV) is a virus from the *Flavivirus* genus. In humans this virus is transmitted mainly by *Aedes aegypti* mosquitoes. However, recent evidences indicate the occurrence of sexual transmission. Although some studies have indicated testes and prostate as the main organs that collaborate in this transmission, little is known about which cell types in these tissues are more susceptible to this virus. In addition, infection with distinct ZIKV strains in some models *in vitro* and *in vivo* has demonstrated that the host's response to infection is strain-dependent. Recent findings suggest that this virus deregulates host miRNA profile and that this is an important event throughout the course of the infection. Herein, we evaluated the susceptibility, the permissiveness and the cellular miRNA profile of human prostatic epithelial cells (PNT1A) to two different strains of ZIKV, a classical African strain, MR766 ($ZIKV^{MR766}$) and a Brazilian strain, $ZIKV^{BR}$. So, we infected PNT1A cells with ZIKV strains and performed an indirect immunofluorescence assay for protein envelope; monitored infectious viral particles production and RNA viral copies by plate assay and qPCR, respectively, and analyzed the miRNA cellular profile by PCR array. Our results demonstrated that human prostate cells are susceptible and permissive to ZIKV infection and did not present any imposition regarding infection by distinct strains of this virus. The strains did not differ in the kinetics of replication in prostate cells, but presented differences in miRNA expression modulation. After infection, 16 miRNAs were modulated in prostate cells, a small group of 6 miRNAs were modulated by both strains while a set of 10 miRNAs showed to be modulated exclusively by $ZIKV^{BR}$. *In silico* analyses predicted that the miRNA upregulated exclusively by the infection by the Brazilian strain may regulate genes and pathways associated to inflammation, immunity, cell survival and cell proliferation. Taken together, our results indicate that prostate may be an important role in the sexual transmission of ZIKV and highlights that different strains of ZIKV may induce a differential host miRNA expression which may influence the differences in the physiopathology presented after the infection by different strains.

Keywords: Prostate. Sexual transmission. miRNAs. Zika virus Brazilian strain. MR766 strain. Zika virus.

3.1 Introduction

Zika virus (ZIKV), a member of *Flaviviridae* family was isolated in 1947 in Zika Forest, Uganda [1]. ZIKV circulation was restricted to Africa and Asia until 2007 and the most of infection were asymptomatic or presented mild symptoms, such as fever, rash, headache, myalgia, conjunctivitis and join pain [2-4]. Three outbreaks occurred after the worldwide spread of ZIKV: in Yap Island, Micronesia (2007), French Polynesia (2014) and Brazil (2015). The epidemic in French Polynesia and Brazil emerged with neurological complications, like the Guillain-Barre Syndrome(GBS) and Zika Congenital Syndrome, not reported previously [5-9].

ZIKV is divided into two main lineages: African and Asian. The strains circulating in Brazil descend from Asian ZIKV [10]. Some studies demonstrated that pathology effects of ZIKV infection may be strain-dependent. Studies based of *in vitro* and *in vivo* models has shown that African lineages are more aggressive than Asian strains [11, 12]. There are few studies comparing differential virulence among ZIKV lineages and most of them analyzed these strains in the nervous system [12-15].

Recent reports documented sexual transmission of ZIKV [16-19]. Most of the cases involves male-to-female transmission, but male-to-male and female-to-male transmission have also been reported [18, 20, 21]. It is possible that only a small part of sexual transmission has been reported since at least half of ZIKV infections are asymptomatic [5, 22-24]. The interaction between ZIKV and human urogenital tract remains unclear, but there are evidences that this virus replicates in reproductive organs. Zika virus RNA was persistent in semen up to 370 days after the onset of symptoms [25] and infectious viral particles were detectable up to 69 days after the symptoms onset [26].

Early studies evaluating the kinetics of ZIKV in male reproductive tract have indicated the testicle and epididymis as potential organs for viral replication in urogenital tract [27, 28]. However, the presence of ZIKV RNA in semen of infected-vasectomized man [26], the high viral load in prostate of mice and monkeys infected with ZIKV, a prostatitis in *Rhesus* monkeys and a high susceptibility and permissiveness of human prostate cells suggest that prostate is a potential replication site and reservoir for ZIKV in humans, and may contribute for the maintenance of the virus in semen [29-31].

An important event in *Flavivirus* infection is the alteration of gene expression, as a result of controlling antiviral cellular responses or to subvert the intracellular environment for better viral

replication [32]. In this process, the cellular microRNAs (miRNAs) profile may be altered. Several *Flavivirus* infection resulted in changes of host miRNAs expression, which were associated with antiviral activity [33-35]. Zika virus infection in liver, lung and kidney cells modulated the expression of AGO2, DICER and DROSHA genes, key proteins in miRNA maturation pathway [36]. Astrocytes infected with ZIKV showed alterations of miRNAs expression associated with viral life cycle, viral transcription, viral processes, cell cycle, innate immune response and unfolded protein response pathway [37]. These evidences indicate that miRNA regulation is an important event in Zika virus infection. However, no study to date has compared if different ZIKV lineages modulate differentially the host miRNA profile in reproductive male tract cells. As such, understanding the miRNA modulation among ZIKV lineages and its impact on infection of prostate cells is essential to improve our understanding of *Zika virus* sexual transmission.

Here we infected human prostate cells with African and Brazilian ZIKV strains to investigate if these cells are susceptible and permissive to ZIKV. We evaluated than if they display differences in infectivity and miRNA modulation.

3.2 Material and methods

3.2.1 Cell Culture

PNT1A (Human prostate epithelial cells, non-tumoral) and Vero clone E6 cells (kidney African Green Monkey) were grown in high-glucose Dulbecco's modified eagle medium, DMEM, (Cultilab, São Paulo, Brazil) supplemented with 10% fetal bovine serum (FBS) (Cultilab, São Paulo, Brazil), 1000 U/mL penicillin-streptomycin, P/S (Thermo Fisher Scientific, Massachusetts, United States of America) in a humidified incubator at 37°C with 5% CO2. Clone C6/36 cell line (*Aedes albopictus*, Banco de células do Rio de Janeiro, BCRJ, Brazil) was grown in Leibovitz L-15 medium, L-15 (Cultilab, São Paulo, Brazil) supplemented with 10% FBS (Cultilab, São Paulo, Brazil) and 1% P/S (Thermo Fisher Scientific, Massachusetts, United States of America) in an incubator at 28°C.

3.2.2 Virus preparation

Two strains of Zika virus were used in this study. The Brazilian ZIKV strain, $ZIKV^{BR}$, was isolated from a febrile patient in the state of Paraiba [38] and it was kindly provided by Dr. Pedro Vasconcelos, Instituto Evandro Chagas, Brazil. The African ZIKV strain, $ZIKV^{MR766}$ was courtesy

of Dr. Amilcar Tanuri, Universidade Federal do Rio de Janeiro, Brazil. For viral propagation, a sample of these virus was inoculated in C6/36 cells and incubated for 5-6 days until the onset of visible cytopathic effects. Subsequently, a freeze-thaw cycle was done, the supernatant was centrifuged, filtrated, aliquoted and stored at – 80°C. The characterization of these strains was done by titration using plaque assay in Vero cells.

3.2.3 Plaque assay

Vero cells were seeded ($2x10^5$) in each well of a 12-well plate. Virus samples were serially diluted in DMEM (Cultilab, São Paulo, Brazil) without supplementation, added to the plate and incubated for 1 hour at 37°C with 5% CO_2, getting shaken every 5 minutes. Following, the viral inoculum was aspirated and 950 µL of DMEM (Cultilab, São Paulo, Brazil) supplemented with 1% FBS (Cultilab, São Paulo, Brazil) and P/S (Thermo Fisher Scientific, Massachusetts, United States of America) and 1% carboxymethylcellulose sodium salt (Sigma Aldrich, Missouri, United States of America) were added to the wells and incubated for 96 hours. Cells were fixed with 1 mL of 10% formaldehyde (Sigma Aldrich, Missouri, United States of America) and stained with 1% crystal violet (Sigma Aldrich, Missouri, United States of America) diluted in 20% ethanol (Merck, Darmstadt, Germany). Plaques were counted from each dilution and infectivity titer express such as plaque forming unit/mL (PFU/mL) was calculated [39].

3.2.4 Indirect Immunofluorescence assay

In order to determine the susceptibility of prostate cells to ZIKV strains, indirect immunofluorescence assay (IFA) for detection of the envelope protein (E) was performed. PNT1A cells ($5x10^4$) were seeded per well of a 96-well plate and incubated at 37°C with 5% CO_2 for 24 hours prior to infection. Approximately 10^5 PFU/mL of $ZIKV^{BR}$, $ZIKV^{MR766}$ in a total volume of 40 µL were added in each well by 1 hour. After infection, the plate was incubated for 72 hours at 37°C with 5% CO_2. Cells controls were performed exactly as for infected cells with conditioned medium from uninfected cells. IFA was performed by fixing $ZIKV^{BR}$ or $ZIKV^{MR766}$ - infected cells to well with 4% paraformaldehyde (Sigma Aldrich, Missouri, United States of America) for 20 minutes and washing 3 times with 1x PBS. Cells were permeabilized with 0.2% PBS/Triton (Merck, Darmstadt, Germany) by 5 minutes at room temperature (RT). Cells were blocked with 3% bovine serum albumin (BSA)/ PBS (Sigma Aldrich, Missouri, United States of America) for

30 minutes at RT and incubated with mouse 4G2 monoclonal antibody (mAB, Millipore, Massachusetts, United States of America) at 1:250 dilution for 2 hours at RT for detection of the E protein of *Flavivirus*. Following, cells were washed 3 times with 1x PBS and incubated with Alexa Fluor 594 - anti-mouse IgG, 1:500 (A21203, Thermo Fisher Scientific, Massachusetts, United States of America) for 1 hour at RT. Cells were washed 3 times and stained with DAPI (1:7000, Sigma Aldrich, Missouri, United States of America), imaging was captured on a Zeiss Axio Vert. A1 microscope.

3.2.5 Viral Permissiveness Assay

In order to analyze if prostate cells are permissive to $ZIKV^{BR}$ and $ZIKV^{MR766}$ PNT1A cells were seeded in 6-well plate (10^6 cells/well) and incubated at 37°C with 5% CO_2 for 24 hours prior to infection. Approximately 10^5 PFU/mL of $ZIKV^{BR}$, $ZIKV^{MR766}$ in a total volume of 700 µL were added in each well by 1 hour. After infection, the plates were incubated for 6, 12, 24, 48, 72 and 96 hours at 37°C with 5% CO_2. PNT1A cells supernatants were collected from each experimental time and it was titled using plaque assay in Vero cells. The assay was performed in duplicate and 3 independent experiments.

3.2.6 Viral RNA copies quantification by qPCR

qPCR was performed to quantify the intracellular and extracellular ZIKV RNA at each time-point. RNA from the supernatants of infected-cell were extracted 6, 12, 24, 48, 72 and 96 hours after infection using Trizol Reagent (Thermo Fisher Scientific, Massachusetts, United States of America) according to manufacturer's instructions. The intracellular RNA from PNT1A cells was extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Massachusetts, United States of America). Absolute quantifications were performed on a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, California, United States of America) using ZIKV 1086, ZIKV 1162c (primers) and ZIKV 1107 (probe) [40] and TaqMan Universal PCR Master Mix, No AmpErase® UNG (Thermo Fisher Scientific, Massachusetts, United States of America).

3.2.7 PCR analysis

The miRNAs profile of prostate cells infected with ZIKV strains were evaluated at the time-point with the highest viral titer. RNA extracted from PNT1A cells as described previously was converted to cDNA using the miScript II RT Kit (Qiagen, Hilden, Germany). miRNAs profile was evaluated by MIHS-001Za-12 miScript miRNA PCR array, Human miFinder (Qiagen, Hilden, Germany), which allows the analysis of 84 miRNAs cellular. The reaction was performed using miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) on a 7300 Real Time PCR System (Applied Biosystems, California, United States of America). Data was analyzed using the ΔΔCT method of relative quantification by the free data analysis software available at pcrdataanalysis.sabiosciences.com/mirna. Each assay was performed in triplicate.

Gene Ontology analyses were performed using DIANA-miRPath v3.0 [41] to identify potentially gene ontology biological process that are enriched by the target genes of miRNAs regulated. The default was defined by *p*-value threshold < 0.05. The total number of gene targets for each miRNA deregulated (>2-fold) in response to $ZIKV^{MR766}$ and $ZIKV^{BR}$ infection were identified using TarBase v.7 and v.8 [42], a database which correlates experimentally verified miRNA targets.

3.2.8 Statistical analysis

All statistical analysis was performed with Prism GraphPad 5.0 (GraphPad Software, Inc., California, United States of America), using t-test paired samples. Statistical significance was defined as for $p < 0.05$.

3.3 Results

3.3.1 PNT1A cells are susceptible to ZIKV strains

To analyze the susceptibility of PNT1A cells to $ZIKV^{BR}$ and $ZIKV^{MR766}$ immunofluorescence assay for envelope protein was performed. Infection was evaluated 72 hours after infection and the results show that PNT1A cell line is susceptible to both ZIKV strains (Figures 1A and 1B).

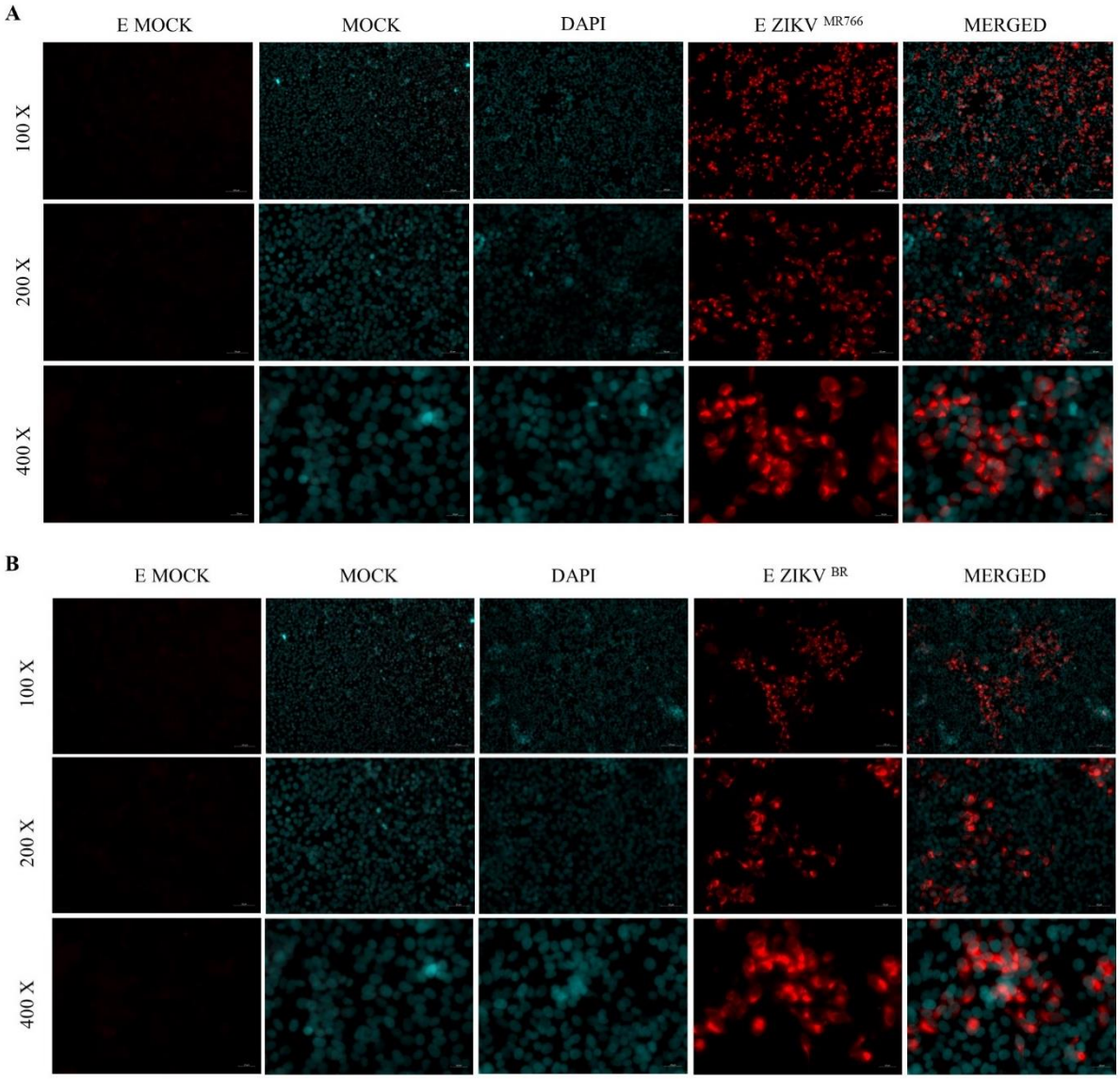

Figure 1: PNT1A cells are highly susceptible to ZIKV^{BR} and ZIKV^{MR766}. Cells were infected with 10^5 PFU/mL of the respective strains for 1 hour. Seventy-two hours after infection cells were fixed with paraformaldehyde and indirect immunofluorescence for mAB 4G2 (red fluorescence) antibody was performed. DAPI was used for staining the nuclei (blue fluorescence). Mock (non-infected cells) was performed with the same procedure used for infected-cells. A) ZIKV^{MR766} infected-cells; B) ZIKV^{BR} infected-cells. E: envelope protein.

3.3.2 Human prostate cells are permissive to ZIKV strains

To evaluate if prostate cells support ZIKV^{MR766} and ZIKV^{BR} replication, PNT1A cells were infected by 10^5 PFU/mL of ZIKV strains during 1 hour and plaque assay and qPCR were performed

to evaluate infectious particles production and total viral particles production, respectively. The production of infectious particles was monitored for up to 96 hours. Both strains showed efficient replication and infectious particles production in prostate cell line after 6, 12, 24, 48, 72 and 96 hours post infection (hpi). The amount of infectious virus increased about 6 times from 6 hpi to 96 hpi for ZIKV^{MR766} and about 4 times under the same conditions for ZIKV^{BR} (Figure 2). ZIKV^{MR766} and ZIKV^{BR} strains did not show statistical difference in the production of infectious viral particles in these cells (p = 0.1039).

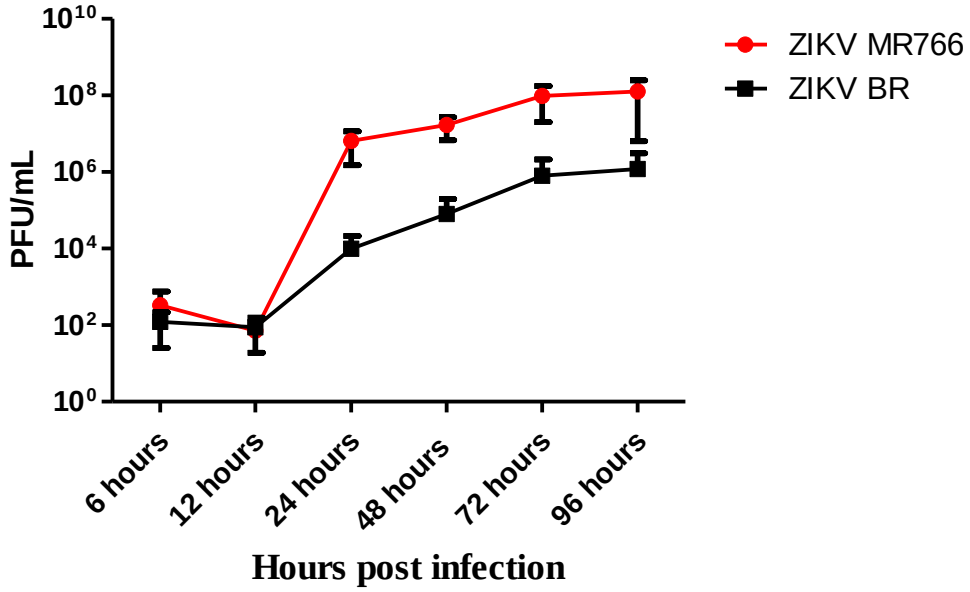

Figure 2: ZIKV replicates efficiently in human prostate cells, producing a high amount of infectious viral particles 96 hours post infection. PNT1A cells were infected with 10^5 PFU/mL of ZIKV^{MR766} or ZIKV^{BR}. Supernatants were collected and tittered by plaque assay after 6, 12, 24, 48, 72 and 96 hpi. The graphic presents the mean ± SD from 3 independent experiments. The means are not statistically significant (p = 0.1039).

The same supernatant tittered by plaque assay was quantified by qPCR to evaluate the total production of viral particles. The supernatant of ZIKV infected-cells showed the same trend of infectious viral particles release, with a higher number of viral copies for ZIKV^{MR766} when compared to ZIKV^{BR}, although not significant (p = 0.2653). The peak of viral copies was observed at 72 hpi and a decrease was observed 96 hpi for both strains (Figure 3).

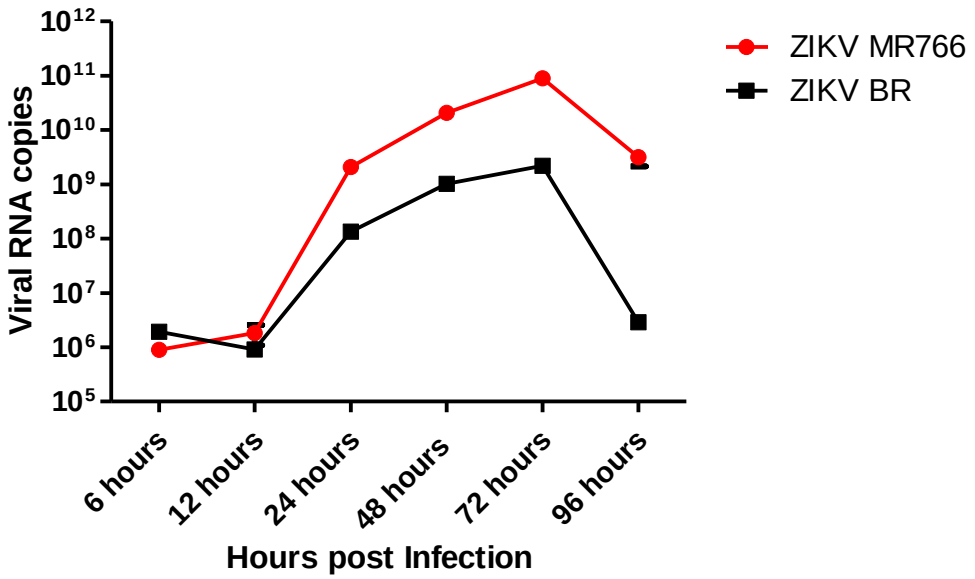

Figure 3: ZIKV replicates efficiently in human prostate cells, releasing a high amount of viral copies. PNT1A cells were infected with 10^5 PFU/mL of $ZIKV^{MR766}$ or $ZIKV^{BR}$. After 6, 12, 24, 48, 72 and 96 hpi supernatants were collected and viral RNA copies were determined by qPCR. The graphic presents the mean ± SD. Differences are not statistically significant ($p = 0.2653$).

The intracellular amount of ZIKV RNA was determined by qPCR. A high amount of intracellular viral copies was observed for both strains with similar values. The peak of infection was observed at 48 hpi for $ZIKV^{MR766}$ and 72 hpi for $ZIKV^{BR}$ (Figure 4).

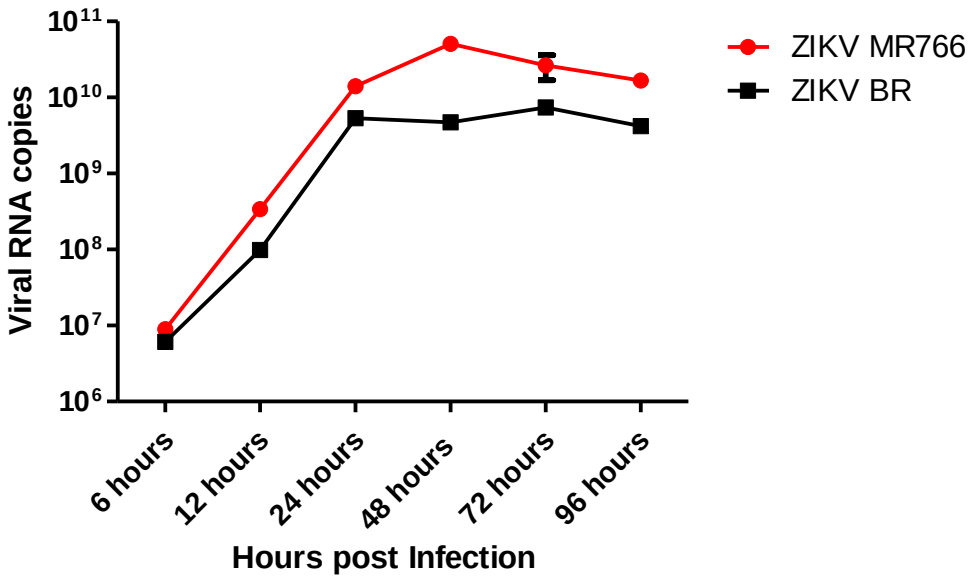

Figure 4: Human prostate cells exhibit the peak of infection at 48 hpi for ZIKV^{MR766} and at 72 hpi for ZIKV^{BR}. PNT1A cells were infected with 10^5 PFU/mL of ZIKV^{MR766} or ZIKV^{BR}. After 6, 12, 24, 48, 72 and 96 of infection cellular lysates were collected and viral RNA copies were determined by qPCR. High amounts of viral RNA copies were observed for both strains, with a peak of infection at 48 hpi for ZIKV^{MR766} and 72 hpi for ZIKV^{BR}. The graphic presents the mean ± SD. Differences are not statistically significant (p = 0.0949).

The ZIKV^{MR766} strain showed a higher rate of infectious particles released in relation to the total amount of particles produced than ZIKV^{BR} in most time-points (Figures 5A and 5B, respectively). Both strains did not differ statistically between the total particles released in relation to infectious particles, p = 0.2119 (ZIKV^{MR766}) and p = 0.1864 (ZIKV^{BR}).

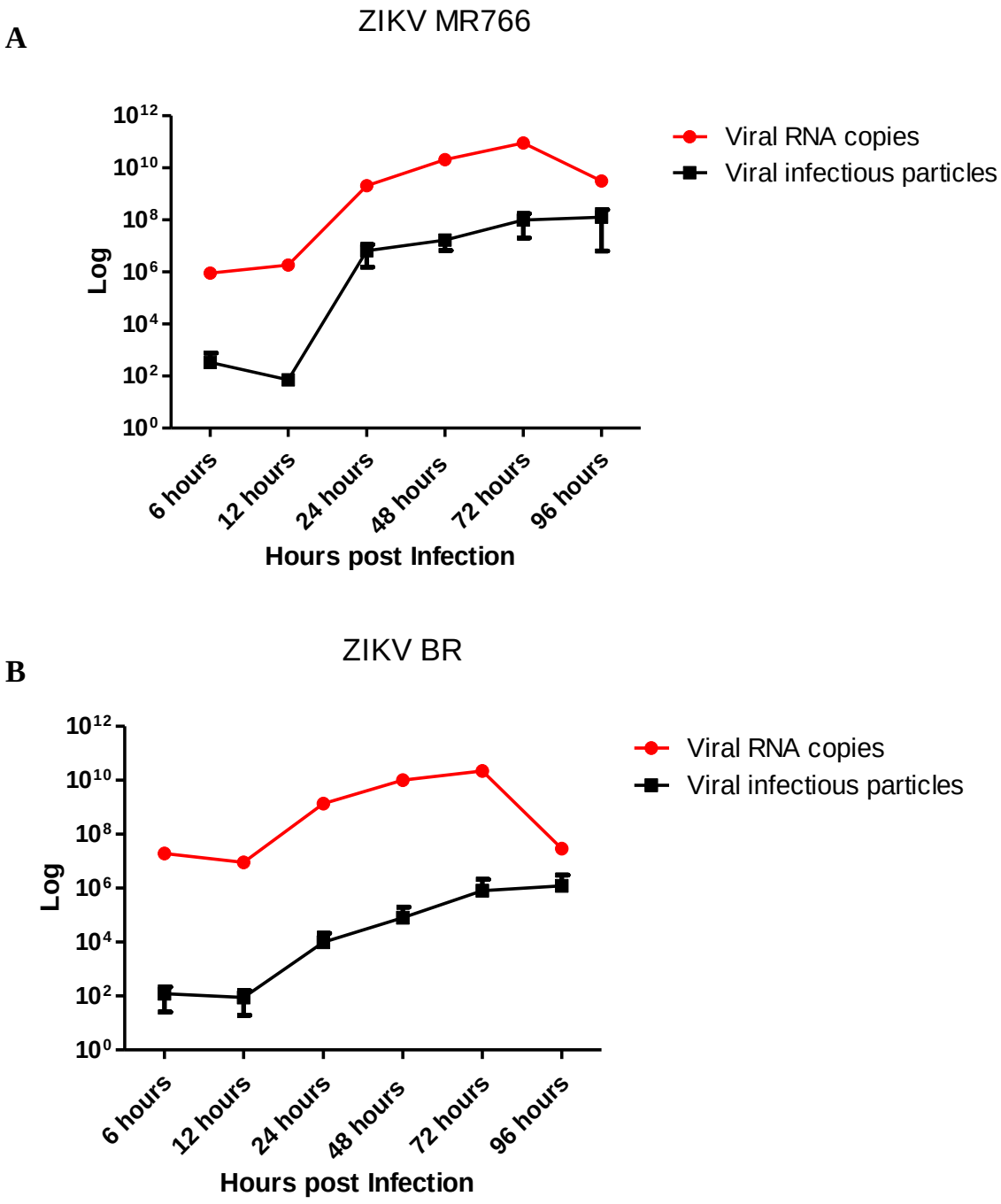

Figure 5: ZIKV^{MR766} presented higher production of viral infectious particles than ZIKV^{BR} in human prostate cells in most infections times. PNT1A cells were infected with 10^5 PFU/mL of ZIKV^{MR766}. After 6, 12, 24, 48, 72 and 96 of infection supernatants were collected and tittered by plaque assay and viral RNA copies were determined by qPCR. ZIKV^{MR766} showed greater production of infectious particles in relation to total particles released than ZIKV^{BR}. The graphic presents the mean ± SD. A) Total particles released in relation to infectious particles after infection

by ZIKV^{MR766}. The means are not statistically significant (p = 0.2119). B) Total particles released in relation to infectious particles after infection by ZIKV^{BR}. The means are not statistically significant (p = 0.1864).

3.3.3 ZIKV^{BR} infection altered an exclusive set of cellular miRNAs

In order to investigate the effect of different ZIKV strains on cellular miRNA expression, we analyzed the differential expression of 84 miRNAs targets by PCR Array. Based on the results from the analysis of intracellular viral load we determined to evaluate the miRNA expression at 48 hpi (the peak of infection) by both strains as well as in control uninfected cells. After ZIKV infection, sixteen miRNAs were differentially expressed by more than 2-fold: six miRNAs after ZIKV^{MR766} infection and sixteen miRNAs after ZIKV^{BR} infection (Table 1). The same set of six miRNAs deregulated in ZIKV^{MR766} infection was also modulated in ZIKV^{BR} infection. However, a set of 10 miRNAs were only modulated in response to ZIKV Brazilian strain infection (Figure 6). In addition, miR-150-5p was downregulated in ZIKV^{MR766} infection and upregulated in ZIKV^{BR} infection (Table 1), taken together, these results suggest that these strains differentially modulate the cell response.

Table 1: List of deregulated miRNAs in prostate cells after ZIKV infection.

	Fold change	
miRNA ID	**ZIKV^{MR766}**	**ZIKV^{BR}**
hsa-miR-150-5p	-3.8194	2.3674
hsa-miR-101-3p	7.0453	7.7097
hsa-miR-99a-5p	2.042	5.2537
hsa-miR-143-3p	5.0982	3.9724
hsa-miR-302a-3p	2.5669	6.379
hsa-miR-22-3p	-4.3873	-7.8717
hsa-miR-142-5p		5.2659
hsa-miR-9-5p		3.4502
hsa-miR-302b-3p		5.2659
hsa-miR-223-3p		3.6553
hsa-miR-210-3p		-2.2921
hsa-miR-141-3p		5.2659
hsa-miR-144-3p		5.2659
hsa-miR-122-5p		5.7891
hsa-miR-302c-3p		5.2659
hsa-miR-142-3p		5.2659

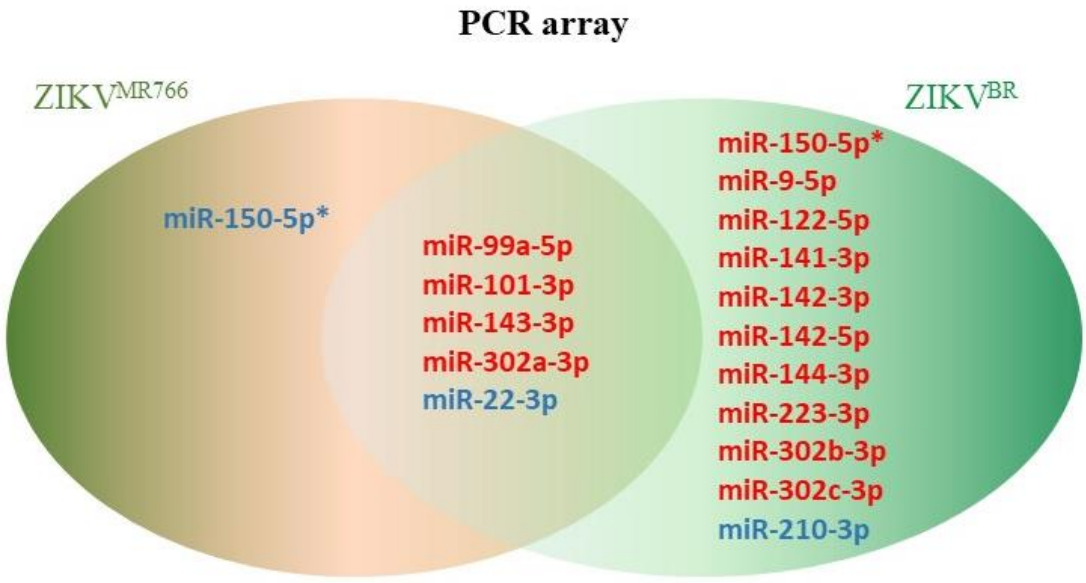

Figure 6: **Differential expression of cellular miRNAs in prostate cells after infection with different strains of ZIKV.** In red color are miRNAs upregulated and in blue color are miRNAs downregulated. *miR-150-5p was downregulated in ZIKV^{MR766} infection and upregulated in ZIKV^{BR} infection.

3.3.4 *In silico* prediction of target genes of miRNAs differentially expressed and pathways potentially enriched by the target genes

In order to identify potential target genes for miRNAs differentially expressed in prostate cells, we performed an *in silico* search for interactions experimentally validated among each miRNA and their genes target. Using the TarBase v.07 database, we identified 10.200 predicted interaction between miRNAs and its gene targets for the 16 miRNAs deregulated in our study (Table S1). After that, we performed a bioinformatics analyses using the mirPath v.03 to correlate pathways potentially affected by miRNAs (Table S2, S3 and S4). Among the pathways identified are pathways associated to cell cycle, immune response, apoptosis, cell grown and survival. Interestingly, some potential pathways enriched for the subset of miRNAs regulated after ZIKV Brazilian strain infection are involved in regulation of key processes of inflammation, immunity and survival, and was the focus of our work. They were NF-kappa B signaling pathway (KEGG hsa04064, Figure 7); TNF signaling pathway (KEGG 04668, Figure 8), PI3K-Akt signaling pathway (KEGG 04151, Figure 9) and Apoptosis (KEGG 04210, Figure 10). In these pathways we

investigated the target genes common to the largest number of miRNAs with differential expression after infection with $ZIKV^{BR}$ (Table 2).

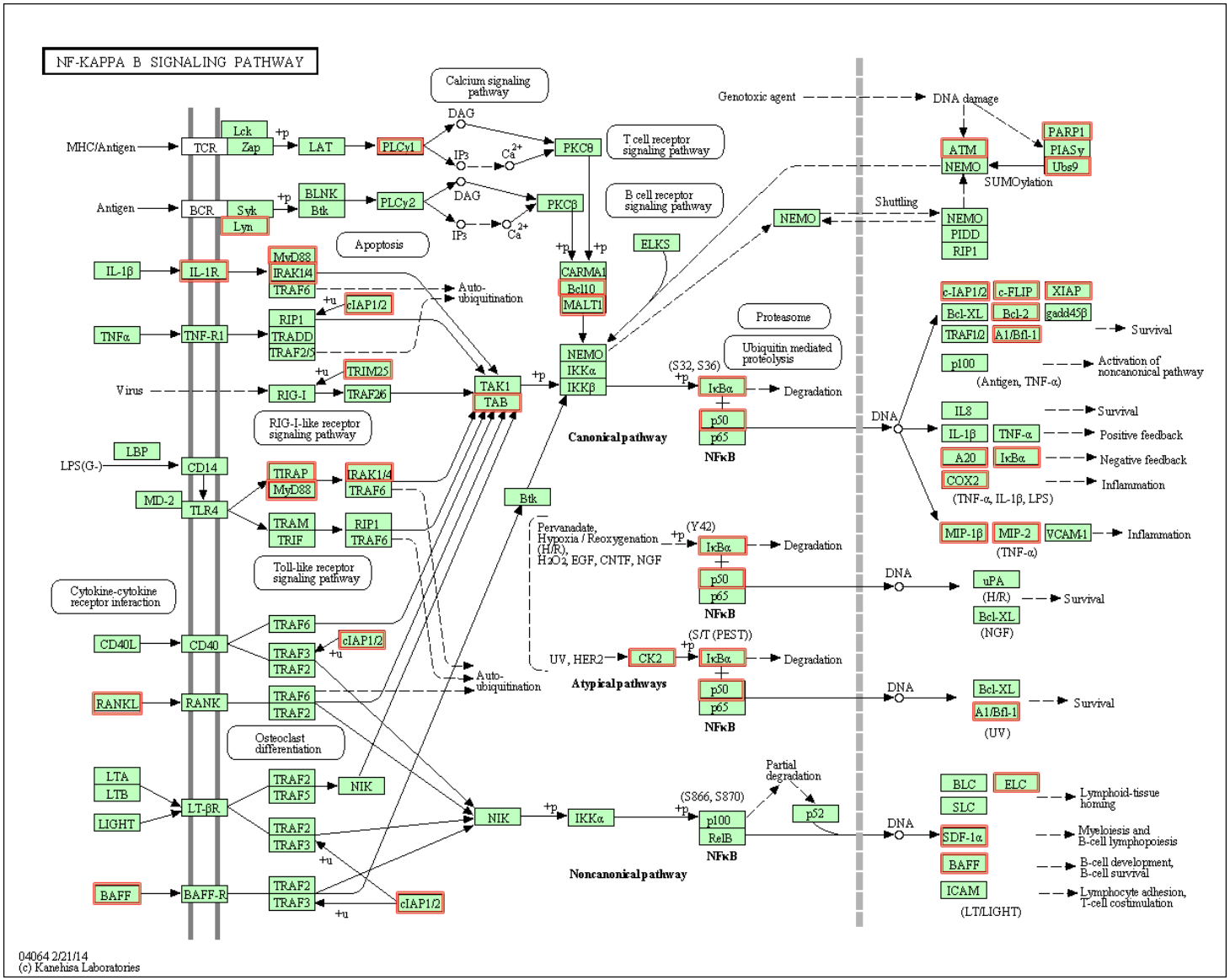

Figure 7: *In silico* prediction of NF-kappa B signaling pathway enriched in ZIKV^{BR} infection. The target genes of the miRNAs associated with this pathway are highlighted.

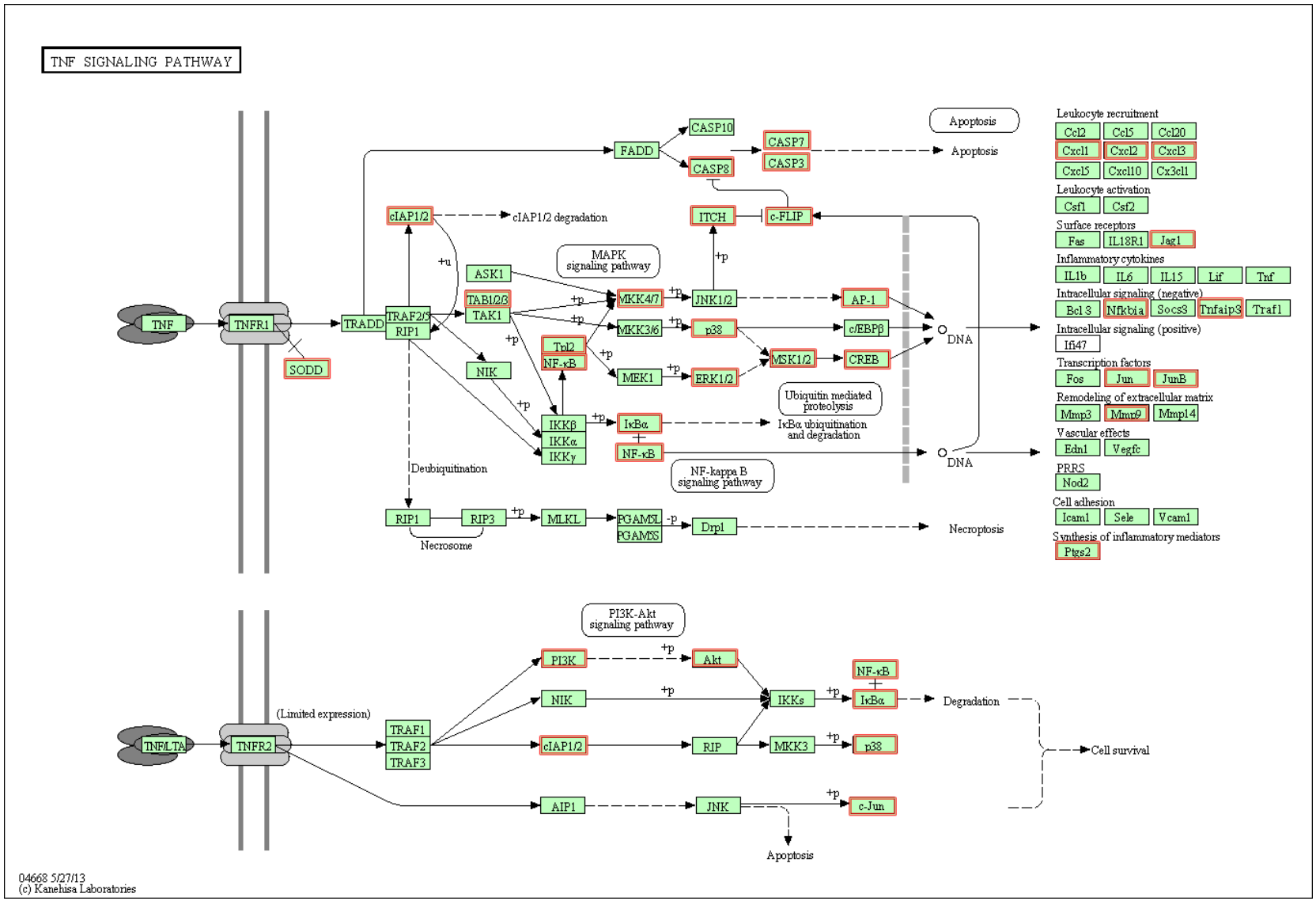

Figure 8: *In silico* prediction of TNF signaling pathway enriched in ZIKV^{BR} infection. The target genes of the miRNAs associated with this pathway are highlighted.

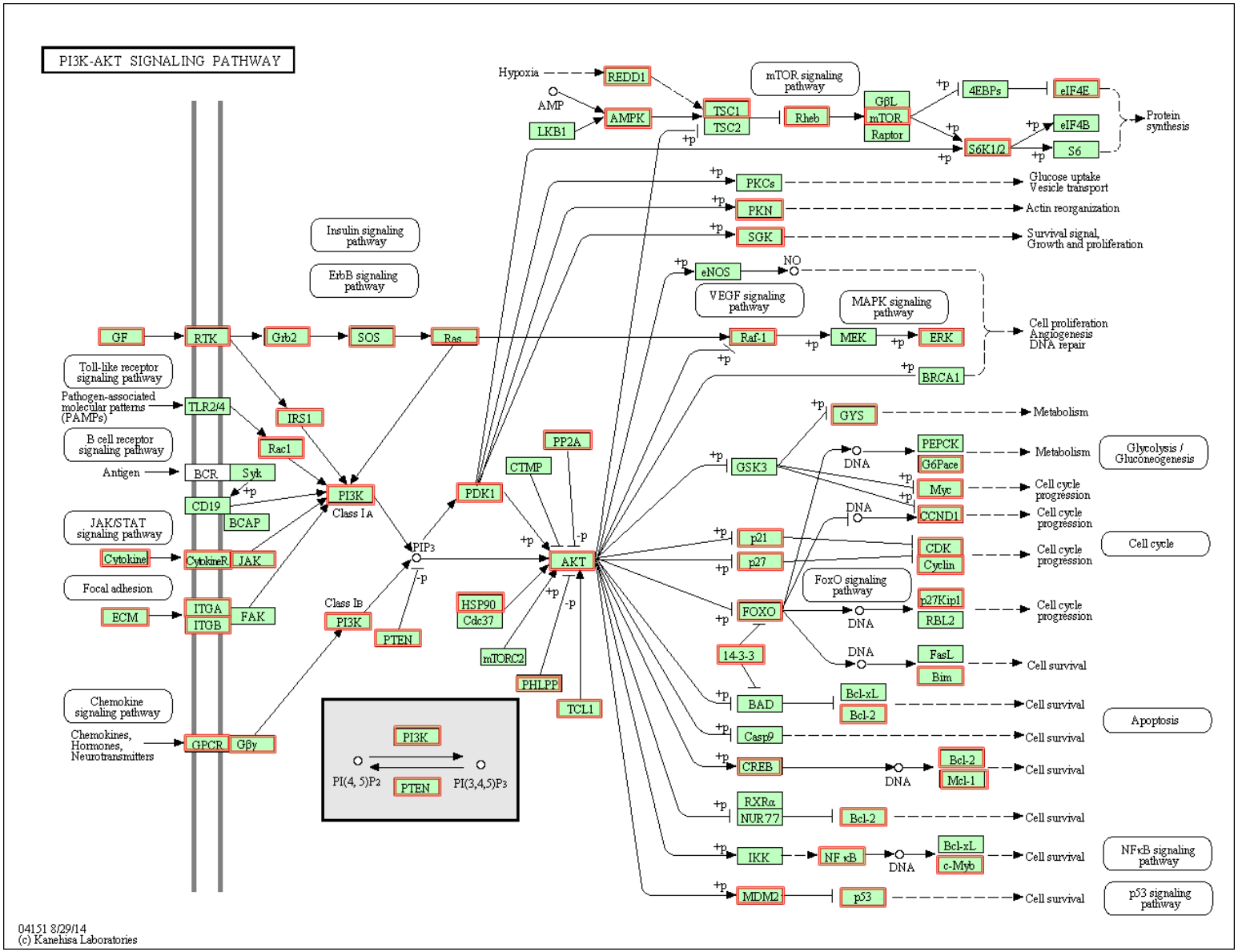

Figure 9: *In silico* prediction of PI3K-AKT signaling pathway enriched in ZIKV^{BR} infection. The target genes of the miRNAs associated with this pathway are highlighted.

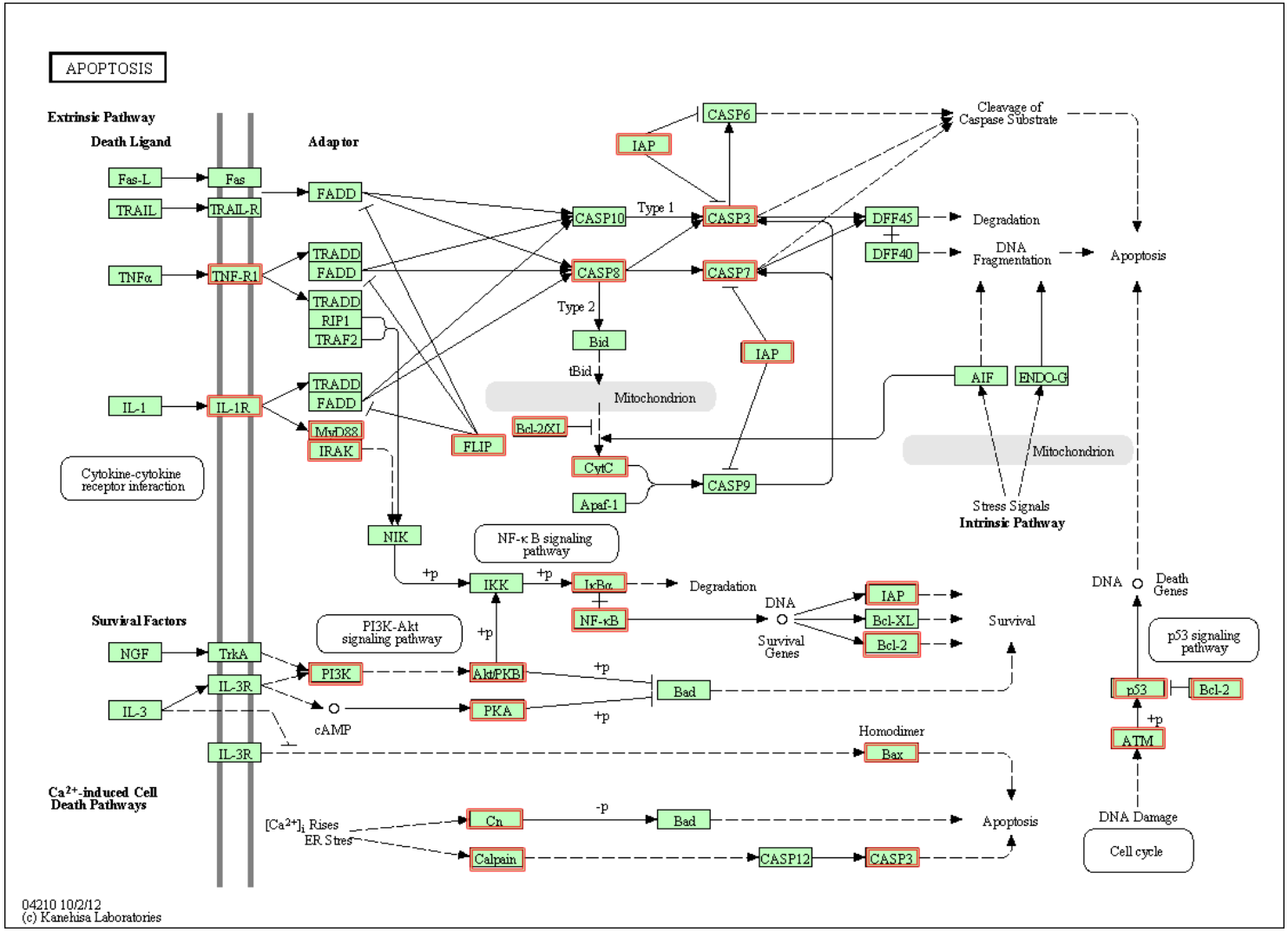

Figure 10: *In silico* prediction of Apoptosis signaling pathway enriched in ZIKV^{BR} infection. The target genes of the miRNAs associated with this pathway are highlighted.

Table 2: *In silico* prediction of pathways enriched in ZIKV^{BR} infection. The target genes of the miRNAs associated with these pathways are highlighted. The genes illustrated in this table are target genes of more than three miRNAs differentially expressed in the PCR array.

Pathway	Genes	miRNAs
NF-kappa B signaling pathway	***ATM***	miR-9-5p; miR-142-3p, miR-122-5p
	PTGS2	miR-9-5p; miR-142-3p; miR-141-3p
	CFLAR	miR-9-5p; miR-142-3p, miR-122-5p
TNF signaling pathway	***PTGS2***	miR-9-5p; miR-142-3p; miR-141-3p
	MAPK1	miR-9-5p; miR-142-3p; miR-142-5p
	AKT1	miR-122-5p; miR-302b-3p; miR-302c-3p
PI3K-Akt signaling pathway	***CCND1***	miR-141-3p; miR-142-5p; miR-9-5p; miR-302c-3p; miR-302b-3p
	CCND2	miR-141-3p; miR-144-3p; miR-302c-3p; miR-302b-3p
	MCL1	miR-141-3p; miR-144-3p; miR-142-5p; miR-302c-3p
Apoptosis	***ATM***	miR-9-5p; miR-142-3p, miR-122-5p
	PIK3CD	miR-122-5p; miR-142-3p; miR-223-3p
	AKT1	miR-122-5p; miR-302b-3p; miR-302c-3p

3.4 Discussion

The first ZIKV reports date of Zika Forest, Uganda, in 1947 [1]. In the years that followed up to 2007 outbreak in Micronesia, ZIKV infection usually was asymptomatic or presented mild symptoms, such as fever, rash, joint pain, conjunctivitis and myalgia [44]. However, ZIKV outbreaks in French Polynesia in 2014 and Brazil, 2015 emerged with neurological complications [7-9].

New pieces of evidence also suggested sexual transmission of ZIKV [18, 20, 21, 45]. The presence of ZIKV RNA in semen of infected men [46, 47] indicates that urogenital tissues are

potential reservoirs and viral repositories. Furthermore, detection of ZIKV RNA in semen of a vasectomized man indicated that prostate may constitute an important replicative site in human male tract [48]. In this study a ZIKV classical strain, MR766 and a strain circulating in Brazil, $ZIKV^{BR}$ were used to analyze their potential to infect human prostate cell line and modulate the host cell miRNAs profile with the aim to verify if the different ZIKV strains present difference in their infectivity. We have demonstrated that human prostate cells are susceptible to $ZIKV^{MR766}$ and $ZIKV^{BR}$ infection, evidenced by the high rate of envelope-protein 72 hpi. Two other prostate cell lines, 19I prostate stromal and LNCaP prostate epithelial adenocarcinoma were also reported to be susceptible to three ZIKV strains (FLR, FLA and HN16). Furthermore, a prostate organoid assembled from this cells demonstrated a tropism of this virus to epithelial cells and its capacity to infect complexes structure [31]. This high susceptibility of prostate cells to get infected by different ZIKV strains corroborates that prostatic tissue is a potential ZIKV reservoir in the human male genital tract. Additionally, ZIKV strains used in our study replicates efficiently in PNT1A cells, releasing more than 10^8 RNA copies after 24 hours of infection. Infectious particles production was time-dependent and reached titles higher than 10^8 PFU/mL four days after infection. The intracellular amount of ZIKV RNA and infectious particles released here were higher than values observed in lung and kidney cells and similar to results of Vero and Huh 7.5, cell lines well established in *Flavivirus* researches, infected by a Polynesia strain, [49]. These higher values suggest a better adaptation and replication of ZIKV in human genital tract cells than lung or kidney cells, contributing to the sexual transmission of ZIKV [21, 26].

A clinical study observed the presence of infectious virus in semen of 8 men infected by ZIKV for up to 38 days after symptoms onset when viral load was $5.7x10^6$ RNA copies/mL [47]. The most probable tissues to produce ZIKV in the human genital tract are the prostate and testes [50]. Our results demonstrated that human prostatic tissue is a potential replicative site for ZIKV in the human genital tract, producing a high amount of infectious virus, which can contribute with high ZIKV load viral observed in semen of infected men. Moreover, our results support that in vasectomized men the prostate would be the only source of ZIKV in semen [26, 48].

The amount of infectious viral particles produced from $ZIKV^{MR766}$ infected prostate cells were 30-fold higher than that produced by $ZIKV^{BR}$ infected cells, but not significant. Although we did not observe a significant difference in viral particles production, analysis of the cellular miRNA

profile in prostate cells showed a differential modulation of miRNAs after infection by $ZIKV^{BR}$ when compared to $ZIKV^{MR766}$ infection.

Our findings suggest that ZIKV induces an upregulation of miRNAs in prostate cells, with a small subset being downregulated. Differently of our results, ZIKV infection in human astrocytes induced a global downregulated of miRNAs, with a small subset being upregulated [37]. Besides that, the set of miRNAs upregulated in prostate cells differs from that upregulated in astrocyte cells, with only hsa-miR-9-5p being common in both tissue. This difference in the modulation of miRNAs suggests that miRNA ZIKV modulation may be tissue-specific.

The analyses of miRNAs profile in prostate cells after infection by ZIKV strains of different lineages indicate a common regulation of a subset of miRNAs for both strains, but an exclusive group of miRNA are modulated only by $ZIKV^{B}$ infection. In order to investigate the particularities of $ZIKV^{BR}$ infection, we focused the functional biological analyses in the exclusive group of miRNAs regulated after Brazilian ZIKV infection. Interestingly, the miR-150-5p expression was downregulated in $ZIKV^{MR766}$ infection and upregulated in $ZIKV^{BR}$ infection, suggesting that this miRNA may have an important role in ZIKV infection by different strains. Or may be differently regulated due to a response to the other targets that are only modulated by $ZIKV^{BR}$ infection.

The miR-150-5p is widely reported to be involved in the innate and adaptive immune response [54-58] and its main role is associated with regulation of differentiation and maturation of B and T cells [54-56, 58]. However, the level of miR-150 has also been correlated with pathology of some viruses. People infected with the *Influenza A/H1N1 virus*, member of *Orthomyxoviridae* family, showed over-expression of circulating miR-150 in the severe form of the disease, which were positively correlated with expression of *IL-1b, CXCL8, IFN-ϒ* and *G-CSF*. Thus, the authors concluded that up-regulation of miR-150 may contribute to the dysregulated pro-inflammatory phenotype in severe A/H1N1 infection [59].

In *Human Immunodeficiency Virus* (HIV) infection (*Retroviridae* family) this miRNA mediated the translational repression of viral RNA, through the binding to the 3'UTR of HIV RNA and so, inhibition of translation of almost all viral proteins [60]. In members of *Flaviviridae* family infection, a study showed the enrichment of miR-150-5p and others miRNAs in exosomes plasmatic of *Hepatitis C Virus* (HCV) viremic patients, which are associated with immunoregulatory signaling pathways [61]. In *Japanese encephalitis virus* (JEV) infection, a member of *Flavivirus* genus, phylogenetically closer to ZIKV, miR-150-5p was up-regulated in

cerebrospinal fluid (CSF) of acute encephalitis patients. The miR-150-5p level was negatively correlated with *TNF* level in CSF, suggesting that this miRNA may be involved in JEV-induced pathogenesis [62].

In our study, the miR-150-5p expression was differentially regulated in ZIKV infection. In viruses, this miRNA is mainly associated with cytokine production, inflammatory and immune responses [59, 62] so, its levels may be involved with different phenotypes of infection by different strains of ZIKV [12, 13, 63].

Using *in silico* analyses we predicted the pathways that the miRNAs upregulated in $ZIKV^{BR}$ infection may regulate. These pathways may provide an indication of what could be altered by infection by Asian strains. Given the potential role of the prostate in the sexual transmission of the *Zika virus* [31, 50, 64] we chose among the pathways enriched those with key functions that can help elucidate the possible relationship of ZIKV with this organ and the sexual transmission. These pathways are involved in immunity, inflammation, cell survival and apoptosis, key processes during *Flavivirus* infection [65, 66]. In this pathways, we discuss about those genes that are targets of more than three differentially expressed miRNAs, because they have a greater potential to be regulated after $ZIKV^{BR}$ infection in prostate cells.

The nuclear factor kappa B (NF-kappa B) is a family of transcription factor that regulates the expression of genes involved in innate and adaptive immunity, inflammation, stress responses, B-cell development, and lymphoid organogenesis [67]. In *West Nile virus* infection (WNV), member of *Flavivirus* genus, the NF-kappa B signaling was activated in monocyte-derived macrophage (MDM) in response to WNV and was required for induction of IDO (indoleamine 2,3-dioxigenase), an antiviral protein. IDO expression was more pronounced in uninfected, neighboring cells in exposed culture to WNV, so, its activity were associated to limit the spread of WNV [68]. In JEV infection, the NF-kappa B pathway is associated to generate pro-inflammatory and pro-survival signals, through to act such key transcriptional factors of proinflammatory cytokines [69, 70]. Differentially, in *Dengue* virus (DENV) infection, the NF-kappa B was associated to enhanced viral replication and viral infectivity [71].

ATM (ATM serine/threonine kinase) is a serine/threonine protein kinase codified by *ATM* gene involved with DNA double strand break and oxidative stress [72]. This protein kinase coordinates several cell responses after DNA damage or it mediates pathways that regulate the redox homeostasis. After an increase of reactive oxygen species (ROS) ATM may indirectly

interfere in mTORC1 signaling, enhancing autophagy [72]. Oxidative stress is a process of ROS formation in the cell. *Flaviviridae* infection like HCV, JEV and DENV induce the formation of ROS, which may lead to ATM regulatory activity [65]. ATM/CHK2 pathway was activated in JEV and HCV infections. The protein CHEK2 is activated by ATM in response to DNA damage and the inhibition of both proteins significantly reduced JEV and HCV production, suggesting a beneficial role for ATM/CHEK2 in replication of this virus [73, 74]. The protective role for ATM/CHEK2 in virus replication is associate with the hijack of CHEK2 by viruses, thus elongating the cell cycle arrest and creating a time window for viral replication [73]. In DENV infection, the early ATM activation was necessary for Endoplasmic Reticulum stress response and autophagy activation, protecting the virus-load cells against others cell stressors [75].

PTGS2 (prostaglandin-endoperoxide synthase 2) also known as cyclooxygenase 2 is an inducible isozyme of PTGS, an enzyme responsible for prostaglandins biosynthesis involved in inflammation and mitogenesis [76]. Prostaglandins are key molecule in generation of inflammatory response and in inflamed tissues their biosynthesis are increased, contributing to the development of acute inflammatory response [76]. The level of cyclooxygenase 2 pro-inflammatory mediator were increased early after JEV and may be involved in immunological responses in neurons in response to this virus [77]. Cyclooxygenase 2 also showed increased levels in blood samples from patients with Dengue fever and Dengue hemorrhagic fever, showing correlation between its levels and severity of disease; having its expression induced in hepatoma cells after DENV-2 infection and cyclooxygenase 2 overexpression enhanced DENV-2 replication in hepatoma cells [78].

CFLAR (CASP8 and FADD like apoptosis regulator), is an anti-apoptotic protein. This protein suppresses the caspase -8 and -10 activation, preventing the activation of downstream apoptosis cascade. Besides that, *CFLAR* also activates cytoprotective signaling pathways involved in cell survival, proliferation and carcinogenesis [79]. In *Hepatitis B virus* (HBV) infection (*Hepadnaviridae* family), *CFLAR* is essential for HBV replication, because protects it from ubiquitin-dependent degradation and regulates the expression of hepatocyte nuclear factors, which are essentials for HBV transcription and maintenance of hepatocytes [80]. In addition, *CFLAR* was moderately up-regulated in HCV patients, together with other proteins associated with apoptosis [81].

Another pathway correlated with miRNAs modulated in $ZIKV^{BR}$ infection was TNF signaling pathway. Tumor necrosis factor (*TNF*) is a proinflammatory cytokine involved in cell

proliferation, differentiation and apoptosis, through the activation of several signaling pathways, such as NF-kappa B [82]. Some *Flavivirus* were associated with the regulation of *TNF*. Patients with HCV chronic showed high up-regulation of *TNF* and other apoptosis markers in immune cells, suggesting that this virus modulate a widely set of apoptotic genes [81]. A higher amount of *TNF* was detected in the supernatant of WNV-infected MDM and its expression mediate the induction of IDO, whose function was discussed above [68].

The gene *PTGS2* is involved with inflammation, as mentioned before. Mitogen-activated protein kinase 1 (*MAPK1*), is a member of *MAP* kinase family. This kinase family is involved with cellular proliferation, differentiation, transcription regulation and development. *MAPK1* activity is associated with control of cell proliferation, since its activation is required for G_1/S transition and cell cycle progression [83]. MAPK1 kinase was phosphorylated on DENV infection, increasing apoptosis in hepatocyte, what was associated with DENV-induced liver injury [84] and NF-kappa B transcription factors activation in human umbilical cord vein endothelial cells [85]. This kinase also was phosphorylated in Vero cells in response to *Yellow fever virus* (YFV) vaccine strain, and pharmacological inhibition of MEK1/2 using U0126 blockades the MAPK1 phosphorylation and YFV replication, suggesting that the MEK/MAPK signaling pathway is required for virus replication [86]. Also in WNV infection, the MAPK1 pathway contributes to cell survival after viral infection [87].

The AKT serine/threonine kinase 1 encoded by the *AKT1* gene is a member of AKT kinase family, a set of key proteins regulators that control cellular processes like grown, survival, proliferation and metabolism. *AKT1* is a critical regulator in cell survival, as demonstrated in knockout mouse studies [88]. *AKT1* regulates cell survival for serine and/or threonine phosphorylation of several downstream substrates [89], like as Bcl-2 homology (BH_3) domain-only proteins, regulating negatively the pro-apoptotic protein, promoting cell survival [90]. Few studies were done associating *AKT1* and *Flavivirus*. Human glioma cell line U251 and BV-2 cells (mouse microglial cell line) infected with JEV showed phosphorylation of *AKT1*, *PKA* (protein kinase A) e *MAPK8* (mitogen-activated protein kinase 8) in response to viral infection accompanied by the release of inflammatory cytokines TNF-a, IL-1b, IL-6, and CCL2. Inhibition of *AKT1*, *PKA* e *MAPK8* in these cells significantly reduced the amount of inflammatory cytokines, suggesting that *AKT*, *PKA* and *MAPK8* pathways are involved in the JEV-induced inflammatory response in glial cells [91].

The PI3K-AKT signaling pathway is a key intracellular signal transducing pathway in regulation of many biological processes, such as metabolism, angiogenesis, proliferation, grown and cell survival [92]. The phosphatidylinositol 3-kinase (*PI3K*) and *AKT* are key proteins in this pathway, phosphorylating a range of downstream substrates. In JEV infection PI3K-AKT signaling pathway was activated early during infection [93] and other studies showed that in *Flavivirus* infection early activation of PI3K/AKT pathway during the viral infection is important to maintenance host cell survival and stimulate anti-apoptotic pathways [94, 95]. Already in DENV infection, a subgenomic viral RNA modulates the PI3K/AKT pathway and triggers apoptotic death through a Bcl-2 related mechanism [96].

Cyclin D1 (*CCND1*) and cyclin D2 (*CCND2*) are members of highly conserved cyclin family, acting as cyclin-dependent kinases (*CDK*) regulators in the progression of cell cycle [97, 98]. *CCND1* and *CCND2* promotes the G_1-S phase progression through its binding and activation of *CDK 4* and *CDK 6* and subsequent functional inactivation of retinoblastoma protein, *pRb* [99-101]. In HCV infection, the ARFP/core+1 viral protein increased the levels of *CCND1* and phosphorylated the *pRb*, resulting in cell cycle progression, what might be involved with an enhance of liver carcinogenesis [102]. Similarly, HCV core protein and NS4b promote the expression of *CCND1* in Huh7 cells [103], what also was associated with progression of cell cycle. Thus, cyclin D1 may be an important protein in the pathogenesis of HCV. *CCND2* was showed in acts as a restriction factor of HIV replication in non-proliferating macrophages, through the regulation of cell cycle protein [104]. In addition, a single nucleotide polymorphism in CCND2 was associated with risk of hepatocellular carcinoma (HCC) in patients with chronic HCV infection [105], mainly because it is involved in cell cycle progression.

The MCL1 apoptosis regulator, BCL2 family member (*MCL-1*) is a key anti-apoptotic regulator. This protein inhibit the pro-apoptotic BCL2 proteins, for sequestrate the direct activator proteins (BH3-only proteins) and/or the effectors (Bax/Bak), blocking the mitochondrial outer membrane permeabilization, protecting from intrinsic apoptosis [106]. In human hepatoma cells, the NS4b protein of HCV induced the *MCL-1* expression in *STAT3* manner-dependent. This induction of *MCL-1* and other genes target of *STAT3* pathway was associated to HCC risk during natural HCV infection [107].

The fourth pathway computationally predicted in our study is apoptosis. This pathway is a biological processes controlled by innumerous others pathways. There are two main processes, the

extrinsic pathway and the intrinsic pathway. The extrinsic pathway is controlled mainly by TNF pathway members, such as TNFRI, Fas or TRAILR (death receptors), and their specific ligands, such as TNF-alpha, FasLG or TNFSF10. The intrinsic pathway is activated from non-receptor stimulus, like as DNA damage and cell stress. This pathway results in the mitochondrial outer membrane permeabilization with release of c cytochrome, and participates them proteins such BCL2 family members, like mentioned before [106].

Flavivirus infection have been reported to trigger apoptosis both by the intrinsic and extrinsic pathways [66, 96, 108, 109]. The regulation of apoptosis by *Flavivirus* involves the activity of viral proteins, which might be pro-survival or pro-apoptotic activity. The core protein in DENV was associated to a pro-apoptotic function while in WNV this protein showed pro-survival effects. Also the membrane proteins, envelope, NS2a, NS2b and NS3 were reported with pro-apoptotic function in a wide group of *Flavivirus* and cells lines [66]. The induction or suppression of apoptosis during *Flavivirus* infection is briefly, a consequence of an attempt of host factors to clear infected cells or an action of the virus to achieve its viral propagation [66].

As mentioned before, the ATM kinase is involved with cell response after DNA damage and oxidative stress. After several types of DNA damage, ATM kinase phosphorylates p53, increasing its transcriptional activity [110-112]. In addition, in response to DNA damage, the ATM may simultaneously phosphorylate p53 and the negative regulator of p53, MDM2 proto-oncogene (*MDM2*), enhancing the p53 activity [113]. A study suggests that the cell death induced by ZIKV infection is related to the interaction between ZIKV capsid protein and *MDM2*, which is involved in apoptosis mediated by the p53 pathway [114].

Another gene that participates in this pathway is *AKT1*, which is a positive regulator of pro-apoptotic BCL-2-member proteins, as mentioned before. *PIK3CD* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta) is a member of PI3Ks, a family of intracellular lipid kinases, which are involved in cell metabolism, survival, polarity and vesicle trafficking [92]. The *PIK3CD* encode a highly homologous p110 catalytic subunit isoforms p110δ [115]. This p110δ catalytic subunit when together with a p85 regulatory subunit constitutes a class I PIK3 protein. This class regulates glucose homeostasis, cell migration, growth and proliferation [116]. No study so far has associated the *PIK3CD* with *Flavivirus* infection, but this protein plays a key role in Natural killer cells maturation and effector functions [117]. Besides that, the *PI3KCD* was crucial for *in vivo* production of natural antibodies and for development of B-1 and marginal zone B cells

[118]. Natural antibodies may recognize cross-reactive epitopes on pathogenic viruses and apoptotic cells, providing rapid protection against infection and preventing inflammation [119, 120]. Thus, this isoform might play an important role in immunity of viruses infection.

The hypothesis that ZIKV Asian strain, as Brazilian strain, have acquired significant changes in relation to strains of the African lineage is emphasized by phenotypical differences among ZIKV African lineage and Asian strains [12]. In general, ZIKV African lineages are more aggressive than Asian strains, producing higher titers of the virus and inducing higher levels of cellular apoptosis [12, 13, 63]. As circulating strains in Brazil are descended from Asian strains, our results corroborate the hypothesis that the mutations that ZIKV undergo along time may have resulted in a change in its infectious profile [121].

Changes that ZIKV has undergone along its evolution may have contributed to trigger the recent outbreaks [12, 51]. The lowest virulence and replication rate of ZIKV Asian lineages in relation to African strains is believed to contribute to the chronic infection profile presented by the American strains, which is not observed for African strains [4, 12, 13]. Primitive placental cells are highly susceptible to infection by $ZIKV^{MR766}$ and showed a higher cell lysis in relation to Asian lineage ZIKV infection [122]. The similar behavior was observed *in vivo*. Mice infected with ZIKV African lineages showed different degrees of severity, from lethality (Cambodia strain) and severe neurological symptoms (Senegal strain) while mice infected by Asian strain present recovery from disease symptoms by day 10 (Puerto Rico strain) [123]. Taken together, these data evidence a trend of African lineage ZIKV to trigger cell apoptosis and infection while Asian strains present a profile of chronic infection, and thus, they may lead to complications like Zika Congenital Syndrome and Guillain-Barre Syndrome.

This study was the first to compare the host miRNA profile after infection between different strains of ZIKV. Our results suggest a differential expression of miRNAs after African and Brazilian ZIKV infection, demonstrating the importance of comparison among lineages and strains of ZIKV to understand all spectra of the virus infection. *In silico* analyses indicates that miRNAs upregulated in $ZIKV^{BR}$ infection may modulate pathways involved in cell survival, inflammatory response and cell proliferation. Additionally, the data presented here reinforce that the prostate is a potential organ to act as a repository and viral reservoirs of ZIKV and thus may play an important role in sexual transmission of this virus. Strategies to control ZIKV replication in the prostate are

necessary to avoid the sexual spread of this virus, both in regions that do not present the arthropod vector or in endemic areas.

3.5 Conclusion

Our results indicate that prostate cells are highly susceptible and permissive to $ZIKV^{MR766}$ and $ZIKV^{BR}$. Both strains demonstrated similar intracellular viral load and infectious viral particles production in PNT1A cells. Overall, these data suggest that prostate cells support African and Brazilian ZIKV replication and it does not present differential infectivity to these ZIKV strains. Thus, prostate cells can be an important source of this virus in human male reproductive tract.

However, miRNAs profile analyses in prostate cells suggest a differential expression of miRNAs in $ZIKV^{BR}$ infection, proposing a different regulation of host gene expression in relation to African ZIKV infection. In addition, the expression levels of miR-150-5p in prostate cells were different after infection by these ZIKV strains, suggesting that this miRNA might be an important function in *Zika virus* infection. *In silico* analyses indicates that Brazilian *Zika virus* may affected host pathways involved in cell survival, response inflammatory, immunity and cell proliferation. Additional studies are needed for understand the impact of different strains of ZIKV in host miRNA and gene expression regulation, including those comparing different strains in other cell types, such as reproductive cells. Thus, strategies to avoid sexual transmission of Zika virus may be better planned.

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SUPPLEMENTARY MATERIAL

Table S1: Number of experimentally validated interactions among each miRNA and gene targets by TarBase v.07.

miRNA ID	Number of interactions miRNA:gene target
hsa-miR-150-5p	175
hsa-miR-101-3p	1137
hsa-miR-99a-5p	408
hsa-miR-143-3p	684
hsa-miR-302a-3p	408
hsa-miR-22-3p	1727
hsa-miR-142-5p	543
hsa-miR-9-5p	997
hsa-miR-302b-3p	227
hsa-miR-223-3p	55
hsa-miR-210-3p	0
hsa-miR-141-3p	893
hsa-miR-144-3p	236
hsa-miR-122-5p	1524
hsa-miR-302c-3p	395
hsa-miR-142-3p	791

Table S2: Pathways affected by predicted genes targets of miRNAs regulated after infection with ZIKV^{MR766} and ZIKV^{BR}.

Pathways potentially affected by predicted target genes*	Number of miRNAs involved **	Number predicted targets genes of miRNAs: total number of genes associated to pathway (% of genes)***
Cell cycle (hsa04110)	6	60 : 124 (48%)
p53 signaling pathway (hsa04115)	6	32 : 72 (44%)
Adherens junction (hsa04520)	6	31 : 72 (43%)
Pathogenic Escherichia coli infection (hsa05130)	6	23 : 55 (41%)
Central carbon metabolism in cancer (hsa05230)	6	26 : 65 (40%)
Viral carcinogenesis (hsa05203)	6	80 : 201 (39%)
Renal cell carcinoma (hsa05211)	6	27 : 69 (39%)
Proteoglycans in cancer (hsa05205)	6	76 : 201 (37%)
Ubiquitin mediated proteolysis (hsa04120)	6	48 : 137 (35%)
Prion diseases (hsa05020)	6	15 : 35 (34%)
Hippo signaling pathway (hsa04390)	6	53 : 154 (34%)
Transcriptional misregulation in cancer (hsa05202)	6	64 : 186 (34%)
Protein processing in endoplasmic reticulum (hsa04141)	6	57 : 165 (34%)
Epstein-Barr virus infection (hsa05169)	6	67 : 201 (33%)
FoxO signaling pathway (hsa04068)	6	44 : 132 (33%)
RNA transport (hsa03013)	6	54 : 165 (32%)
Focal adhesion (hsa04510)	6	65 : 199 (32%)
Chronic myeloid leukemia (hsa05220)	6	25 : 76 (32%)
TGF-beta signaling pathway (hsa04350)	6	28 : 90 (31%)
Oocyte meiosis (hsa04114)	6	39 : 125 (31%)
Signaling pathways regulating pluripotency of stem cells (hsa04550)	6	44 : 139 (31%)
Rap1 signaling pathway (hsa04015)	6	62 : 206 (30%)
Regulation of actin cytoskeleton (hsa04810)	6	62 : 213 (29%)
Lysine degradation (hsa00310)	6	17 : 59 (28%)
Wnt signaling pathway (hsa04310)	6	44 : 158 (27%)

Colorectal cancer (hsa05210)	6	24 : 86 (27%)
Hepatitis B (hsa05161)	6	45 : 163 (27%)
Endocytosis (hsa04144)	6	64 : 244 (26%)
Arrhythmogenic right ventricular cardiomyopathy (ARVC) (hsa05412)	6	19 : 72 (26%)
Pathways in cancer (hsa05200)	6	123 : 526 (23%)
Shigellosis (hsa05131)	5	23 : 65 (35%)
Glycosaminoglycan biosynthesis - keratan sulfate (hsa00533)	3	6 : 14 (42%)
Fatty acid biosynthesis (hsa00061)	3	2 : 13 (15%)

* Pathways are targeted by at least one miRNA out of the miRNA group regulated after ZIKV infection.

** Number of miRNAs differentially expressed after ZIKV infection whose target genes participate in the pathway.

*** Proportion and percentage of the predicted target genes compared to total pathway-associated genes according KEGG pathway database.

Table S3: Pathways affected by predicted targets of miRNAs regulated exclusively after infection with $ZIKV^{BR}$.

Pathways potentially affected by predicted target genes*	Number of miRNAs involved **	Number predicted targets genes of miRNAs: total number of genes associated to pathway (% of genes) ***
Central carbon metabolism in cancer (hsa05230)	9	31 : 65 (47%)
Renal cell carcinoma (hsa05211)	9	33 : 69 (47%)
Prostate cancer (hsa05215)	9	45: 97 (46%)
Small cell lung cancer (hsa05222)	9	41 : 93 (44%)
FoxO signaling pathway (hsa04068)	9	57 : 132 (43%)
Endometrial cancer (hsa05213)	9	25 : 58 (43%)
Glioma (hsa05214)	9	32 : 75 (42%)
Fc gamma R-mediated phagocytosis (hsa04666)	9	39 : 91 (42%)
Neurotrophin signaling pathway (hsa04722)	9	50 : 119 (42%)
Proteoglycans in cancer (hsa05205)	9	83 : 201 (41%)
Signaling pathways regulating pluripotency of stem cells (hsa04550)	9	57 : 139 (41%)
RNA transport (hsa03013)	9	68 : 165 (41%)
AMPK signaling pathway (hsa04152)	9	50 : 120 (41%)
Melanoma (hsa05218)	9	30 : 72 (41%)
HIF-1 signaling pathway (hsa04066)	9	41 : 100 (41%)
Prolactin signaling pathway (hsa04917)	9	28 : 70 (40%)
T cell receptor signaling pathway (hsa04660)	9	39 : 101 (38%)
Thyroid hormone signaling pathway (hsa04919)	9	44 : 116 (37%)
Insulin signaling pathway (hsa04910)	9	51 : 137 (37%)
Measles (hsa05162)	9	49 : 132 (37%)
Viral carcinogenesis (hsa05203)	9	74 : 201 (36%)
Acute myeloid leukemia (hsa05221)	9	24 : 66 (36%)
Hepatitis B (hsa05161)	9	55 : 155 (35%)
Focal adhesion (hsa04510)	9	70 : 199 (35%)
Regulation of actin cytoskeleton (hsa04810)	9	73 : 213 (34%)
TNF signaling pathway (hsa04668)	9	38 : 110 (34%)

Ubiquitin mediated proteolysis (hsa04120)	9	47 : 137 (34%)
NF-kappa B signaling pathway (hsa04064)	9	32 : 95 (33%)
Colorectal cancer (hsa05210)	9	28 : 86 (32%)
Transcriptional misregulation in cancer (hsa05202)	9	56 : 186 (30%)
Endocytosis (hsa04144)	9	72 : 244 (29%)
PI3K-Akt signaling pathway (hsa04151)	9	106 : 354 (29%)
Phosphatidylinositol signaling system (hsa04070)	9	27 : 99 (27%)
MAPK signaling pathway (hsa04010)	9	82 : 295 (27%)
Pathways in cancer (hsa05200)	9	136 : 526 (25%)
Apoptosis (hsa04210)	9	33 : 136 (24%)
mTOR signaling pathway (hsa04150)	9	27 : 153 (17%)
Adherens junction (hsa04520)	8	38 : 72 (52%)
Chronic myeloid leukemia (hsa05220)	8	38 : 76 (50%)
p53 signaling pathway (hsa04115)	8	35 : 72 (48%)
Bacterial invasion of epithelial cells (hsa05100)	8	35 : 74 (47%)
Bladder cancer (hsa05219)	8	19 : 41 (46%)
RNA degradation (hsa03018)	8	34 : 79 (43%)
Pathogenic Escherichia coli infection (hsa05130)	8	23 : 55 (41%)
Non-small cell lung cancer (hsa05223)	8	27 : 66 (40%)
Pancreatic cancer (hsa05212)	8	30 : 75 (40%)
Cell cycle (hsa04110)	8	47 : 124 (37%)
TGF-beta signaling pathway (hsa04350)	8	32 : 90 (35%)
Hippo signaling pathway (hsa04390)	8	55 : 154 (35%)
mRNA surveillance pathway (hsa03015)	8	31 : 91 (34%)
Sphingolipid signaling pathway (hsa04071)	8	39 : 118 (33%)
Hepatitis C (hsa05160)	8	45 : 155 (29%)
Lysine degradation (hsa00310)	8	15 : 59 (25%)
Shigellosis (hsa05131)	7	28 : 65 (43%)
Dorso-ventral axis formation (hsa04320)	6	14 : 27 (51%)
Steroid biosynthesis (hsa00100)	6	8 : 19 (42%)
N-Glycan biosynthesis (hsa00510)	6	15 : 49 (30%)

Other types of O-glycan biosynthesis (hsa00514)	5	12 : 22 (54%)
Glycosaminoglycan biosynthesis - keratan sulfate (hsa00533)	5	5 : 14 (35%)

* Pathways are targeted by at least one miRNA out of the miRNA group regulated after ZIKV infection.

** Number of miRNAs differentially expressed after ZIKV infection whose target genes participate in the pathway.

*** Proportion and percentage of the predicted target genes compared to total pathway-associated genes according KEGG pathway database.

Table S4: Pathways affected by predicted targets of miR-150-5p.

Pathways potentially affected by predicted target genes*	**Number predicted targets genes of miRNAs: total number of genes associated to pathway (% of genes) ****
Renal cell carcinoma (hsa05211)	5 : 69 (7%)
Adherens junction (hsa04520)	5 : 72 (7%)
Glycosaminoglycan biosynthesis - keratan sulfate (hsa00533)	1 : 14 (7%)
Central carbon metabolism in cancer (hsa05230)	4 : 65 (6%)
HIF-1 signaling pathway (hsa04066)	6 : 100 (6%)
TGF-beta signaling pathway (hsa04350)	5 : 90 (5%)
Viral carcinogenesis (hsa05203)	7 : 201 (3%)
MicroRNAs in cancer (hsa05206)	6 : 299 (2%)
Arrhythmogenic right ventricular cardiomyopathy (ARVC) (hsa05412)	2 : 72 (2%)
Hepatitis B (hsa05161)	4 : 163 (2%)

* Pathways are targeted by at least one miRNA out of the miRNA group regulated after ZIKV infection.

** Proportion and percentage of the predicted target genes compared to total pathway-associated genes according KEGG pathway database.

4. CONSIDERAÇÕES FINAIS

As células prostáticas epiteliais humanas foram suscetíveis e altamente permissivas à infecção por ZIKV e não demonstraram suscetibilidade ou permissividade diferencial a cepa clássica africana do ZIKV, MR766 e a uma cepa brasileira, ZIKV^{BR}. A análise do perfil de miRNAs celulares em células prostáticas humanas sugere que diferentes cepas do vírus Zika podem regular diferencialmente a expressão de miRNAs celulares, o que pode estar associado com os diferentes fenótipos de infecção relatados para cepas africanas e asiáticas do ZIKV. Além disso, 10 miRNAs celulares foram modulados exclusivamente após infecção pela cepa ZIKV^{BR}, sugerindo que esse vírus pode modular diferentes alvos celulares em relação a cepas africanas. O nível de expressão do miR-150-5p foi diferencialmente modulado após infecção pelas cepas do ZIKV analisadas, o que sugere que esse miRNA pode apresentar uma importante função na infecção por cepas distintas do ZIKV.

Análises *in silico* sugerem que a cepa brasileira do Zika vírus pode afetar vias hospedeiras envolvidas na sobrevivência celular, resposta inflamatória, imunidade e proliferação celular. Por fim, nossos resultados indicam que a próstata é um potencial órgão para atuar como reservatório e repositório viral no sistema genital masculino durante a infecção pelo ZIKV, e que seu perfil de miRNAs celulares é diferencialmente modulado após infecção por cepas distintas desse vírus. Estudos adicionais são necessários para compreender o impacto de diferentes linhagens do Zika vírus na regulação de miRNAs celulares e expressão gênica no sistema reprodutor humano.

Nota: "As opiniões, hipóteses e conclusões ou recomendações expressas neste material são de responsabilidade dos autores e não necessariamente refletem a visão da FAPESP".

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