UNIVERSIDADE FEDERAL DE PERNAMBUCO

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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

DARLENE PAIVA BEZERRA

Análise *in vitro* da co-expressão e efeito do microRNA-9 (miR-9-5p) em genes ligados à Calcificação Cerebral Primária Familiar

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Orientador: Prof. Dr. João Ricardo Mendes de Oliveira

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Aprovada em: 28/07/2017

COMISSÃO EXAMINADORA

_____

Prof. Dr. Eduardo Isidoro Carneiro Beltrão
Depto. de Bioquímica – UFPE

_____

Prof. Dr. Leucio Duarte Vieira Filho
Depto. de Fisiologia e Farmacologia - UFPE

_____

Profa. Dra. João Ricardo Mendes de Oliveira - Presidente
Depto. de Neuropsiquiatria - UFPE

Dedico este trabalho aos meus pais
(Denison e Alcineide) e minha irmã Débora por me apoiarem
e serem imprescindíveis na minha formação; Ao meu marido
Tarcísio pelo seu companheirismo e carinho.

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RESUMO

Calcificação Cerebral Primária Familiar (CCPF) é uma doença neurológica caracterizada por depósitos de fosfato de cálcio nos gânglios basais e outras regiões cerebrais sendo frequentemente associada com alterações motoras e comportamentais. Mutações nos genes SLC20A2, PDGFB, PDGFRB e XPR1 estão associadas à CCPF e são preditos como alvo para alguns agentes reguladores, como os microRNAs (miRNAs). MiRNAs são pequenas sequências de RNAs não codificantes que regulam negativamente a expressão gênica no nível pós-transcricional. O MiRNA-9 (miR-9) é um tipo de miRNA seletivamente expresso em tecidos neuronais que desempenha um papel essencial no desenvolvimento neuronal e sabidamente regula a expressão de PDGFR-beta em cardiomiócitos. Nosso objetivo foi observar a influência do miR-9-5p no desenvolvimento ou aprimoramento da calcificação *in vitro*, focado nos genes ligados à CCPF. Um estudo *in silico*, utilizando os softwares online TargetScan e DianaTools, foi feito para verificar se o miR-9-5p regula genes envolvidos na CCPF. As células utilizadas no modelo in vitro foram as SaOs-2 (osteosarcoma) e HEK293 (embrionária renal) foram mantidas em DMEM (Dulbecco's Modified Eagle's medium) suplementado com 10% de soro bovino fetal, 0,5% de glutamina 200 mM e 10,000 U/mL de penicilina/estreptomicina. O meio osteogênico é composto do meio de manutenção adicionado de ácido ascórbico 250 μM e 1 mMβ-glicerofosfato como modelo para a calcificação. O meio das células cultivadas foi substituído a cada 3 dias, exceto o ácido ascórbico que foi adicionado todos os dias. A identificação e quantificação de marcadores de osteoblastos (coloração de citoquímica – Alizarin Red) foram realizadas nos dias 0, 7 e 14. Verificamos a co-expressão do o miR-9-5p em genes envolvidos na CCPF através da transfecção em células durante 24hutilizando lipofectamina 2000.O processo de calcificação e co-expressão foram analisados por qPCR e western blotting. Foi feito um estudo eletrofisiológico para verificar a funcionalidade do miR-9-5p em células SaOs-2. Observamos que durante o processo de calcificação o perfil de expressões de genes envolvidos na CCPF é alterado. Com 14 dias de calcificação, comprovado com o Alizarin Red, o miR-9-5p mostrou uma tendência a aumentar com a proteína PiT2 (SLC20A2) diminuída, comprovando a regulação predita. Com o aumento da expressão do o miR-9-5p, observamos uma significante diminuição do PiT2 e PDGFrB. O uso de um inibidor de o miR-9-5p confirmou os achados prévios. Dados adicionais eletrofisiológicos mostraram a atuação, após 24h de transfecção, do miR-9-5p alterando o perfil nas correntes de entradas e saídas de membrana, mostrando uma atuação mais efetiva via canais de potássio e sódio dependente de voltagem. Concluímos que o miR-9-5p é um importante regulador de genes envolvidos no CCPF, durante a calcificação, podendo ser uma via de estudo para entender melhor o processo de calcificação e um potencial alvo terapêutico.

Palavras-chaves: CCPF. Calcificação. MicroRNA-9. SaOs-2. HEKS293.

ABSTRACT

Primary familial cerebral calcification (PFCC) is a neurological disease characterized by deposits of calcium phosphate in the basal ganglia and other brain regions. This clinical condition has been seen as associated with neurobehavioral and cognitive impairment. Mutations in SLC20A2, PDGFB, PDGFRB and XPR1 genes are associated with PFCC. and are targets predicted to be modulated by many agents, including MicroRNAs (miRNAs). miRNAs are small molecules of non-coding RNAs that negatively regulate post-transcriptional gene expression. MiRNA-9 (miR-9) is a type of miRNA selectively expressed in tissue neurons that plays an essential role in neuronal development at also regulates a PDGFR-beta expression in cardiomyocytes. Our objective was to observe an influence of miR-9-5p on the development and enhancement of *in vitro* calcification. *In silico* study, using the online softwaresTargetScan and DianaTools, was done to check if the miR-9-5p is predicted to regulate genes linked to PFCC. SaOs-2 (osteosarcoma) and HEK293 (embryonic kidney) cells were maintained in DMEM (Dulbecco's Modified Eagle's medium) supplemented with 10% fetal bovine serum, 0.5% glutamine 200 mM and 10,000 U / ml Penicillin / streptomycin. The environment with maintenance medium compound added with 250 μM ascorbic acid and 1 mMβ-glycerophosphate for the calcification model. The medium of the cultured cells was replaced every 3 days except ascorbic acid which was added every day. Identification and quantification of osteoblast markers (cytochemical staining - Red Alizarin) were performed on days 0, 7 and 14. We found a co-expression of miR-9-5p in genes involved in PFCC through transfection in cells for 24 hours using lipofectamine 2000. The calcification and co-expression process were analyzed by qPCR and western blotting analysis. It was an electrophysiological study to verify the functionality of miR-9-5p in SaOs-2 cells. We observed that during the process of calcification the profile of genes expression involved in PFCC was altered. With 14 days of calcification, stained with Alizarin Red, miR-9-5p showed a tendency to increase with a decreased PiT2 (SLC20A2) protein, proving a predicted regulation. With increased expression of miR-9-5p, we observed a significant decrease in PiT2 and PDGFrB. The use of a miR-9-5p inhibitor confirmed the previous findings. Additional electrophysiological data showed that, 24hours after of transfection, miR-9-5p altered the electrophysiological profile in the inputs and outputs currents, aiming at a more effective modulation through potassium and sodium channels voltage-dependent. We conclude that miR-9-5p is an important regulator of genes involved in PFCC during calcification and may be a pathway of study for the understand the calcification process and a potential therapeutic target.

Keywords: PFCC. Calcification. MicroRNA-9. SaOs-2. HEKS293.

LISTA DE FIGURAS

sequências reguladoras miR-9 e miR-9*. Informações obtidas de http://www.mirbase.org/. Fonte: Produção do próprio autor.

LISTA DE TABELAS

LISTA DE ABREVIAÇÕES

CCPF	Calcificação Cerebral Primária Familiar
SLC20A2	Do Inglês, *Solute Carrier Family 20 Member 2*
PDGFRB	Receptores Do Fator De Crescimento Derivado Das Plaquetas
PDGFB	Fator De Crescimento Derivado Das Plaquetas
XPR1	Do Inglês, *Xenotropic And Polytropic Retrovirus Receptor 1*
RNA	Ácido Ribonucleico
PI	Fosfato Inorgânico
SLC34	Do Inglês, *Solute Carrier Family 34*
SLC17	Do Inglês, *Solute Carrier Family 17*
PIT2	Transportador De Fosfato Inorgânico Tipo 2
PIT1	Transportador De Fosfato Inorgânico Tipo 1
KO	Nocautes
LC	Líquido Cefalorraquidiano
RTK	Receptores De Tirosina Quinase
BH	Barreira Hematoencefálica
PLCY	Fosfolipase C-Γ
MLV	Do Inglês , *Murine Leukemia Virus*
SPX	Complexo SYG1/Pho81/XPR1
SYG1	Do Inglês, *Suppressor Of Yeast Gpa1*
PHO81	Do Inglês, *Phosphate System Positive Regulatory Protein*
RNAM	RNA Mensageiro
DROSHA	Do Inglês, *Drosha Ribonuclease Iii*
DGCR8	Do Inglês, *Microprocessor Complex Subunit DGCR8*
RAN	Do Inglês, *Ras-Related Nuclear Protein*
GTP	Guanosina Trifosfato
TRBP	Do Inglês, *Transactivation Responsive RNA Binding Protein*
AGO	Argonauta
RISC	Complexo De Silenciamento Induzido Por Rna
SNC	Sistema Nervoso Central
NPCS	Células Progenitoras Neurais
HEK293	Do Inglês, *Human Embryonic Kidney 293 Cells*

DNA	Ácido Desoxirribonucleico
AD5	Adenovírus Tipo 5
SaOs-2	Do Inglês, *Osteosarcoma Cell Line*
OPG	Osteoprotegerina

SUMÁRIO

1 INTRODUÇÃO

Calcificação Cerebral Primária Familiar (CCPF) também conhecida como "doença de Fahr", é um distúrbio neuropsiquiátrico, caracterizado por depósitos de cálcio nos gânglios basais e outras áreas do cérebro, visualizado por neuroimagem. As calcificações são insidiosas e se expandem progressivamente através dos vasos e parênquima. Esta condição clínica tem sido frequentemente associada com alterações neurocomportamentais e cognitivas. A maioria dos indivíduos afetados permanece durante grande parte da vida aparentando ter uma vida saudável. Apenas entre a quarta e quinta década de vida começam a aparecer graduais e progressivos distúrbios neuropsiquiátricos e de movimento, apesar de haver casos pediátricos. Em pacientes portadores de CCPF, foi observada a presença de um padrão de herança autossômica dominante, recentemente relatada a várias famílias portadoras de mutações no gene *SLC20A2*. CCPF é geneticamente heterogênea, geralmente herdada de forma autossômica dominante. Mutações em quatro genes diferentes foram identificadas como causadores das calcificações cerebrais familiar primárias: *SLC20A2, PDGFRB PDGFB* e *XPR1* (LEGATI et al., 2015) (ARTS et al., 2015).

MicroRNAs (MiRNAs) são reguladores da expressão gênica em muitos processos biológicos, incluindo desenvolvimento, proliferação, apoptose e resposta de stress. São uma classe de pequenos RNAs não-codificantes, composto por 21-23 nucleotídeos. MiRNAs regulam a estabilidade ou a eficiência translacional do RNA mensageiro alvo através da interação complementar com a região 3' não traduzida dos genes alvo (ZIMMERMAN; WU, 2011). MiRNA-9 (miR-9) é seletivamente expresso em tecidos neurais, onde desempenha um papel essencial no desenvolvimento, proliferação e diferenciação neuronal (COOLEN; KATZ; BALLY-CUIF, 2013).

Grande parte do conhecimento atual sobre doenças neurológicas foi adquirida a partir de estudos pós-morte devido às limitações para obtenção de tecido cerebral vivo. Diferenças entre espécies dificultam a simulação de doenças neurológicas humanas em modelos animais. Portanto, a modelagem de doença simulando o fenótipo da doença *in vitro* e em populações celulares é um importante avanço e tornaria possível compreender os mecanismos celulares e moleculares de desordens neurodegenerativas.

O objetivo desse trabalho foi observar a influência do miR-9-5p no desenvolvimento ou aprimoramento da calcificação *in vitro.*

2 REVISÃO BIBLIOGRÁFICA

2.1 CALCIFICAÇÕES CEREBRAIS FAMILIAR PRIMÁRIAS

O primeiro relato de calcificações vasculares nos gânglios da base foi em 1850 por Delacour, sobre um homem de 56 anos que teve, clinicamente, rigidez e fraqueza dos membros inferiores com tremor, o exame revelou a presença de calcificações bilaterais e esclerose. Bamberger, em 1855, descreveu a histopatologia de calcificações em finos vasos cerebrais, em uma mulher que tinha retardo mental e convulsões (MANYAM, 2005). Fahr descreveu um relato de caso de um homem, possivelmente, com hipoparatiroidismo cuja necropsia revelou calcificação na substância branca com pouca calcificação nos Gânglios basais. O nome de Fahr tornou-se associado com todas as formas de calcificações bilaterais nos gânglios basais e em outras partes do cérebro, apesar de não ter sido o primeiro a descrever calcificação no cérebro nem ter contribuído significativamente para a compreensão desta doença. Fritzsche, em 1924, fez a primeira descrição da condição radiográfica. (MANYAM; WALTERS; NARLA, 2001). O interesse foi atraído novamente para esta doença quando Boller *et al*, em 1977, descreveu nove membros de uma mesma família com calcificações cerebrais familiar idiopáticas (PRESS, 2015).

A nomenclatura "calcificações cerebrais familiar primárias" sofreu uma série de modificações desde seu primeiro nome, em 1939, "Calcificação cerebral simétrica" (MANYAM, 2005), o que causava um certo transtorno na padronização dos casos. Durante algum tempo ficou conhecida como "Calcificação idiopática familiar dos gânglios basais" pois as calcificações mostravam uma certa predileção pelos gânglios da base e advinham de causas desconhecidas. Com a descoberta do primeiro gene envolvido o termo idiopático tornou-se inapropriado, e mutações encontradas no mesmo gene estarem diretamente associadas a calcificações cerebrais, a utilização do termo "calcificações primárias" tornou-se mais apropriada, visto que calcificações secundárias podem ser de origem infecciosas, inflamatórias, tóxicas entre outras (SOBRIDO et al., 2014).

Atualmente o termo mais usual e pertinente na literatura é calcificação cerebral primária familiar (CCPF). CCPF é uma doença neurodegenerativa, herdada de forma

autossômica dominante, caracterizada por calcificação cerebrais simétricas bilaterais detectadas por tomografia computadorizada, predominantemente nos gânglios da base, mas também no cerebelo, tálamo e tronco encefálico, como visto na figura 1 (DE OLIVEIRA; DE OLIVEIRA, 2013). As características clínicas envolvem alterações neurológicas e psiquiátricas, mais comumente são descritos transtornos de movimentos, como parkinsonismo, mas podem ocorrer enxaqueca, psicose, demência e mudanças de humor (LEMOS et al., 2015). Por outro lado, pode ocorrer de forma assintomática, como relatado em 2016, onde uma mulher de 55 anos não possuía nenhum transtorno neurológico ou psiquiátrico, não relatou nenhum sintoma anteriormente, e depois de uma queda foi solicitado um exame de imagem onde foram encontradas calcificações extensas e difusas nos gânglios basais e núcleo denteado (GAGLIARDI et al., 2017).

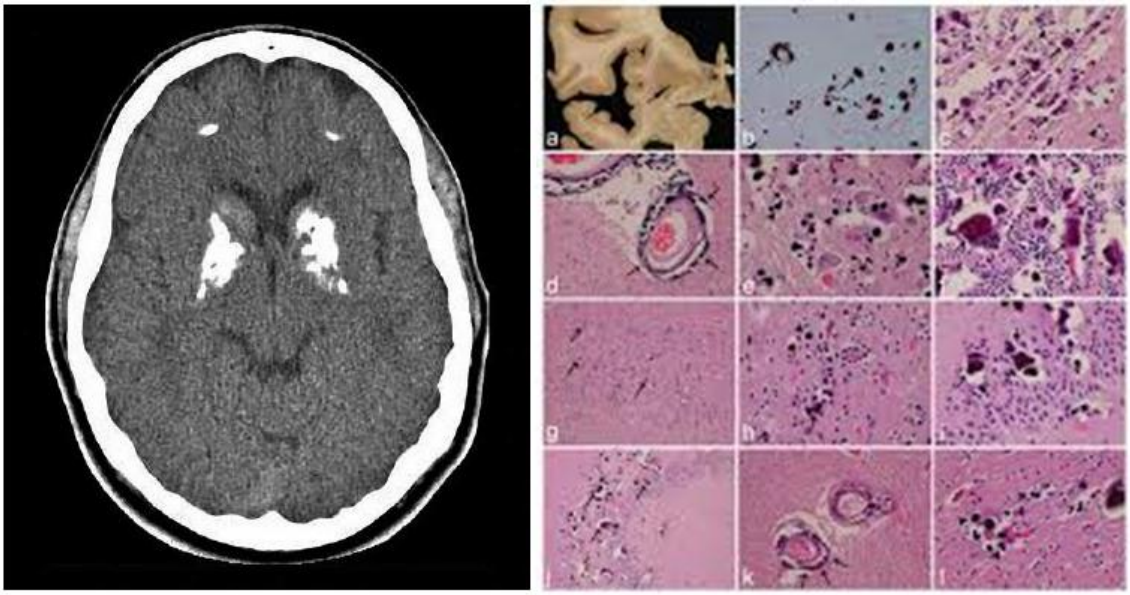


Figura 1 - Padrões de calcificações cerebrais frequentemente encontrados em pacientes com calcificação cerebral primária familiar. Na figura a esquerda, uma imagem obtida a partir de tomografia computadorizada, mostrando a presença de calcinose, seguindo padrão de imagens de indivíduos com CCPF. A direita, uma visão macroscópica (a) mostra descoloração marrom do globo pálido (seta), que tinha uma consistência arenosa na autópsia. Na histologia, as colorações von Kossa (b) e a hematoxilina-eosina (c-l) mostram calcificações generalizadas das regiões profundas do cerebelo (b, c, grande calcificações pleomórficas = seta, calcificação redonda compacta escura = ponta da seta), vasos sanguíneos do cerebelo (d, setas), núcleo denteado (e, calcificação redonda escura = ponta de flecha), córtex do cerebelo (f, grande calcificação pleomórfica = setas), córtex visual (g, h, calcificação pleomórfica = setas, calcificação redonda escura = ponta de flecha), giro denteado do hipocampo (i, j, setas) e substância branca (k, l, calcificação perivascular = flechas, rodada escura calcificação na

proximidade de um capilar = ponta de seta). Fonte: retirado de DE OLIVEIRA; DE OLIVEIRA, 2013 e WIDER et al., 2009, respectivamente.

A calcificação geralmente desenvolve-se dentro da parede do vaso e no espaço perivascular, em última análise, que se estende até o neurônio. Ocorre secundariamente em torno de uma estrutura composta de glicosaminoglicanos e substâncias relacionadas. Progressiva mineralização dos gânglios basais tende a comprimir o lúmen do vaso, iniciando assim uma circulação sanguínea prejudicada, lesão de tecido neural e deposição mineral. Composição mineral das calcificações varia com a localização anatômica e proximidade com as calcificações vasculares. Pode ser devido ao anormal metabolismo de cálcio e fósforo, enquanto alguns relatos tendem a contradizer esta conclusão. Os depósitos são compostos por minerais como carbonato de cálcio e fosfato; gliconato, glicosaminoglicanos e metais incluindo ferro, cobre, magnésio, zinco, alumínio, prata e cobalto (SALEEM et al., 2013).

Os critérios de diagnóstico foram determinados a partir de Moskowitz et al. 1971, Ellie et al. 1989 e Manyam 2005. Entre os critérios podemos destacar: Calcificações cerebrais bilaterais visualizadas em neuroimagem; Disfunção neurológica progressiva, que geralmente inclui uma desordem de movimento e/ou manifestações neuropsiquiátricas; A idade de início na quarta ou quinta década, embora esta disfunção também possa se apresentar na infância; Ausência de alterações bioquímicas e somática; Ausência de uma causa infecciosa e tóxica, ou traumática; Histórico familiar consistente com herança autossômica dominante (MANYAM, 2005; MUFADDEL; AL-HASSANI, 2014).

CCPF é geneticamente heterogênea. Mutações em quatro genes foram identificadas como causadores da CCPF. Em 2012, o primeiro gene foi identificado, o SLC20A2 (Solute Carrier Family 20 Member 2), que codifica um transportador de fosfato inorgânico (Pi) dependente de sódio tipo III (PiT2). Em 2013, os genes PDGFRB, codifica os receptores do fator de crescimento derivado das plaquetas, e PDGFB, que codifica o seu principal ligante, foram também associados ao CCPF. Mutações no gene SLC20A2 leva a um acúmulo de Pi e, subsequentemente, à deposição de fosfato de cálcio. Mutações em PDGFB e PDGFRB também resultam em calcificação, no entanto, por meio de processos indiretos. Recentemente, um quarto gene, XPR1 (Xenotropic And Polytropic Retrovirus Receptor 1), foi mostrado

envolvido na fisiopatologia do CCPF (TADIC et al., 2015). Embora mutações nos quatro genes representem 60% dos casos de CCPF ainda não há uma conhecida via comum de sinalização para formação das calcificações. Até o momento, sabe-se que SLC20A2 e XPR1 estão envolvidos diretamente no metabolismo do fosfato, enquanto PDGFRB e PDGFB estão associados à integridade da barreira hematoencefálica e manutenção de pericitos (OLIVEIRA; OLIVEIRA, 2016) (Figura 2).

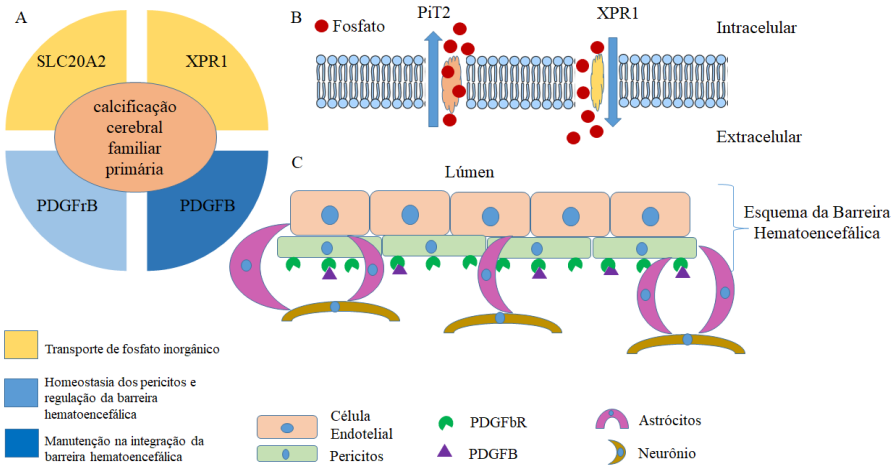


Figura 2 - Representação esquemática de mecanismos responsáveis pela calcificação cerebral. Em A, os genes envolvidos na calcificação cerebral primária familiar e suas vias atuação. Em B, dois genes que codificam transportadores envolvidos diretamente no transporte de fosfato inorgânico, PiT2 e XPR1 agindo em sentidos antagônicos. Em C, uma representação da barreira hematoencefálica (BH), demostrando a presença de componentes celulares (pericitos e células neurais) e o envolvimento de PDGFrB e seu ligante PDGFB na manutenção e sinalização da BH. Fonte: Produção do próprio autor.

2.1.1 *SLC20A2*

O fosfato inorgânico (Pi) é componente biológico estrutural e está envolvido no crescimento, desenvolvimento, formação óssea, regulação ácido-base e metabolismo celular (TO, 2009). A manutenção da homeostase do Pi envolve uma rede de hormônios, fatores de crescimento e tipos celulares (SAPIO; NAVIGLIO, 2015). Nos seres humanos, as reservas de fosfato são distribuídas em 85% nos ossos, 14% em células e 1% em soro e fluidos extracelulares de tecidos moles (BLAINE; CHONCHOL; LEVI, 2015). Diversos fatores podem alterar a regulação dos níveis de fosfato (Figura 3). A manutenção dos níveis fisiológicos Pi total ocorre de forma

altamente regulada através da captação intestinal e reabsorção renal envolvendo famílias de cotransportadores - SLC (solute carrier families): SLC17, SLC34 e SLC20 (tabela1).

Tabela 1 Cotransportadores de fostato inorgânico

Transportador	Local	Envolvimento
SLC17 Cotransportador Na^+/Pi tipo I (NaPi-I)	Fígado e rins	• Transporte transmembranar de ânions orgânicos • Transporte de glutamato vesicular (VGLUT1-3) • Transportador de ácido siálico lisossomal (Sialina).
SLC34 Cotransportador Na^+/Pi tipo II (NaPi-II)	Intestinos e rins	• Absorção e reabsorção de Pi na membrana apical de muitos epitélios.
SLC20 Cotransportador Na^+/Pi tipo III (NaPi-III)	Ampla distribuição tecidual	• Menos bem definidas • Absorção intestinal ; Expresso tanto em tecido epitelial quanto não- epitelial.

A família SLC20 é também conhecida como transportadores Na^+/Pi tipo III (NaPi-III), eletrogênico e simporte onde sódio e fosfato, na proporção 2 : 1, fluem para o meio intracelular. Inicialmente identificados como receptores retrovirais e posteriormente demonstrado serem co-transportadores Na^+/Pi, atualmente representado por duas isoformas, PiT-1 e PiT-2 (SLC20A1, SLC20A2, respectivamente) (INDEN et al., 2013). PiT1 (SLC20A1, Glvr-1 - Leukemia virus receptor 1 homolog) e PiT2 (SLC20A2, Ram-1 - murine amphotropic retrovirus), tem 62% de homologia cuja a organização topológica foi proposta com base em gráficos de índice hidropático, ambos contêm 12 domínios de extensão transmembrana com um único grande domínio intracelular, considerados genes *housekeeping*, já que atuam na manutenção da homeostase do Pi (BØTTGER et al., 2006).

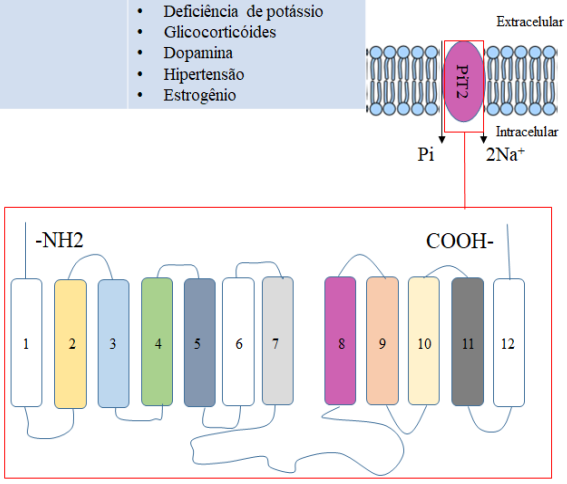
SLC20A2 é localizado no cromossomo 8p.11.21, codifica o transportador de fosfato inorgânico PiT-2. Mutações no SLC20A2 foram as primeiras a serem associadas a CCPF. Inicialmente, Wang et al. (2012) relatou 7 famílias com CCPF da China,

Espanha e Brasil, com diferentes mutações no PiT2 (WANG et al., 2012). Atualmente entre os casos encontrados envolvendo os genes candidatos, representam, aproximadamente, 55% das mutações (75 de 137). Mutações em SLC20A2 são herdadas de forma autossômica dominante. Entre os tipos mutações patogênicas encontradas, há relatos de missense, frameshift, deleções, non-sense, splice site, mutação de novo e grandes deleções genômicas, como observado na figura 3 (LEMOS et al., 2015) (BATLA et al., 2017).

O transporte de fosfato inorgânico é crucial para a homeostasia do cálcio e fosfato celular e a deficiência na função do PiT2 pode contribuir para a deposição de fosfato de cálcio na matriz extracelular vascular. A fisiopatologia da calcificação associada a SLC20A2 foi estudado usando camundongos Slc20a2-nocautes (KO) e modelo celular. Jensen et al. (2013) relatou através de seções cerebrais de camundongos KO (slc20a2 -/-), extensos agregados irregulares bilaterais de esferas calcificadas no tálamo, semelhantes as CCPF, usando coloração com Von Kossa (JENSEN et al., 2013). O KO de Slc20a2 foi suficiente para causar calcificações em camundongos e fortalecer a ligação entre mutações em SLC20A2 e CCPF. Jensen et al. (2015) mostrou aumento nos níveis de fosfato inorgânico (Pi) no líquido cefalorraquidiano (LCR) em camundongos KO (slc20a2 -/-), confirmando que o PiT2 regula Pi no CSF. Essa ideia foi ratificada por Wallingford et al. (2016a, b) que descreveu a importância dessa regulação acrescentando fenótipos centrais e periféricos em camundongos KO incluindo: níveis de fosfato elevado em tecidos produtores de LCR (plexo coróide, células ependimais, músculo liso arteriolar), calcificação de tecido nervoso óptico e hidrocefalia (WALLINGFORD et al., 2016)(WALLINGFORD; GAMMILL; GIACHELLI, 2016). Em células SaOs-2 através de indução química, Keasey et al. (2016), relataram uma diminuição da expressão do PiT2 com a diferenciação osteogênica, confirmada pelo corante alizarin red (KEASEY et al., 2016).

PiT2 é expresso em maiores níveis nas regiões do cérebro afetadas por CCPF, como revelado no Allen Institute Human Brain Atlas (http://www.brain-map.org/) e por ligantes do PiT2 no cérebro de camundongo. Este padrão de expressão pode explicar a vulnerabilidade regional, pois as calcificações vasculares e pericapilar são encontradas apenas na região cerebral.

Aumento na absorção de Fosfato	Diminuição na absorção de Fosfato
• Dieta com baixo teor de fosfato • 1,25- vitamina D3 • Hormônio da tireóide	• Dieta com alto teor de fosfato • Hormônio da paratireóide • Acidose metabólica • Deficiência de potássio • Glicocorticóides • Dopamina • Hipertensão • Estrogênio



Exon	Mutações	Exon	Mutações
2	c.82G>A c.124_126delGTG c.152C>T c.185T>C c.212G>A5 c.260_261delTC c.1753G>A	8	c.935-1G>A c.1086delC c.1101C>G c.1145G>A c.1399C>T c.1409delC c.1470_1478delGCAGGTCCT c.1483G>A c.1492G>A c.1506C>A c.1523+1G>A c.1158C>G c. 1520_1521delTG c.1101_1102delCG c.1301C>G c.1138delC
3	c.344C>T c.338C>G c.323T>C	9	c.1794+1G>A/C c.1652G>A c.1703C>T c.1527delT c.1711G>A c.1527delT c.1618G>A
4	c.514A>T c.515delA c.509delT c.431-1G>T	10	c.1784C>T c.1723G>A c.1711G>A c.1755-1768 delCTCCAACATCGGGC c.1753G>A c.1790G>A
5	c.551C>T c.581A>G c.583_584delGT c.560A>G	11	c.1802C>G/T c.1828_1831delTCCC c.1909A>C
7	c.760C>T c.750delT		

Figura 3 - Organização topológica e mutações no PiT2 e alterações no transporte do fosfato. Na figura acima temos a estrutura do PiT2 com 12 domínios transmembrana, responsável pelo co-transporte eletrogênico de Na^+/ Pi (2:1). Na tabela a esquerda, podem ser observados fatores que alteram a absorção do fosfato. O transporte de fosfato pode ser alterado devido a mutações no PiT2, o principal gene envolvido na calcificação cerebral primária familiar. A tabela a direita mostra as mutações dos tipos missense, frameshift, deleções, non sense, splice site, mutação de novo e grandes deleções genômicas encontradas em pacientes diagnosticados com CCPF. Fonte: Produção do próprio autor.

2 .1.2 *PDGFRB/PDFGB*

PDGFRB (PDGFR-β) e seu principal ligante PDGFB são outros genes envolvido nas CCPF. PDGFB é localizado no 22q13.1, enquanto que PDGFRB no 5q32. Mutações nos genes PDGFB e PDGFRB são responsáveis por 31% e 11% dos casos, respectivamente (BATLA et al., 2017).

PDGFB foi descoberto em 1970 como um importante fator de crescimento sérico de fibroblastos, de células musculares lisas e de células gliais derivado de plaquetas. PDGFs são formados por dímeros de ligações dissulfureto podendo ser composto por quatro cadeias homodiméricas (PDGF-AA, PDGF-BB, PDGF-CC e PDGF-DD) e uma heterodimérica (PDGF-AB). Os PDGFs atuam através de dois receptores de tirosina quinase (RTK) (PDGFRA e PDGFRB)(RAICA; CIMPEAN, 2010).

PDGFRB é crucial para manter a barreira hematoencefálica (BH) e a perda de função devido a presença de mutações pode, potencialmente, levar a permeabilidade alterada em pericitos ao redor dos vasos sanguíneos cerebrais, podendo gerar deposição de cálcio. O ligante PDGFB está envolvido em recrutamento de pericitos, regulação da BH e angiogênese. A perda da regulação do cálcio através da BH, possivelmente, leva a progressiva calcinose (LEMOS et al., 2015). Também foi proposto que as proteínas PDGFs tem funções reguladoras sobre transportadores de fosfato XPR1 e PiT2 no cérebro. As possíveis interações entre os mecanismos de deposição de cálcio relacionado a PiT2, pericitos e BH continuam a serem esclarecidos (LEGATI et al., 2015).

Mutações em PDGFB e PDGFRB seguem o padrão de herança autossômico dominante. Estudos anteriores mostram que densidade reduzida de pericitos está correlacionada com o aumento do diâmetro e redução da densidade de vasos. Genes nocautes de PDGFB ou PDGFRB em camundongos podem causar fenótipos caracterizados por perda de pericitos, hiperplasia endotelial, dilatação vascular, hemorragia, edema e morte no final da gestação ou perinatal (SORIANO, 1994) (KELLER et al., 2013). Mutações parciais em PDGFB ou PDGFRB, por outro lado, são compatíveis com a vida adulta, mas causam disfunção renal e cardiovascular e mudanças neurológicas. Hipoplasia dos pericitos, desenvolvida como uma consequência da redução da sinalização PDGFB/PDGFRB prejudica a maturação da barreira hematoencefálica, durante o período pré-natal, um defeito que persiste na fase adulta. PDGFB é um estimulador potente e seletivo transportador de fosfato inorgânico nas células vascular do músculo liso, um efeito principalmente mediado pelas PLCy (fosfolipase C-γ). Esta possível ligação entre PDGFRB, PLCy e transporte de fosfato merecem uma investigação mais aprofundada, uma vez que mutações no transportador de fosfato inorgânico PiT2 (SLC20A2), e o exportador de fosfato XPR1 também estão ligados a CCPF (DANEMAN et al., 2010)(ARMULIK et al., 2010)(NICOLAS et al., 2013).

2.1.3 *XPR1*

XPR1 é um tipo de receptor retroviral, utilizado por MLV (vírus da leucemia murina) xenotrópico (X-MLV) e politrópico (P-MLV), dois retrovírus que podem

infectar células humanas. XPR1, cuja função permaneceu indescritível por bastante tempo, a partir de estudos de homologia com genes SYG1 de levedura e Pho81 de planta, funcionalmente foi associado a transdução de sinal, e detecção e transporte de fosfato, respectivamente (KOZAK, 2010). Estudos anteriores mostraram, XPR1 foi up-regulado após a ativação de uma via de sinalização (RANKL-RANK) durante a osteoclastogênese (SHARMA et al., 2010). XPR1 está ativamente expresso em células-tronco neurais e cérebros humanos, bem como é expresso em várias regiões do cérebro de camundongos. Envolvimento direto de XPR1 na exportação de fosfato e seu padrão de expressão no cérebro apoiam o seu papel na homeostase do fosfato cerebral (LEGATI et al., 2015).

XPR1 foi o último gene associado a CCPF (LEGATI et al., 2015). São responsáveis por poucos casos relatados (6 casos), e entre os achados clínicos estão parkinsonismo, a disartria e a coreia, até o momento nenhum caso foi descrito com dor de cabeça, distonia ou ataxia cerebral (BATLA et al., 2017). Recentemente, foi mostrado que mutações em XPR1 diminuiu, especificamente, o efluxo de fosfato, como efeito da deficiência de XPR1 (PIMENTEL; LEMOS; OLIVEIRA, 2017).

Tendo em vista a perspectiva das CCPF serem uma doença poligênica e multifatorial, é fundamental estudar mecanismos que regulem genes envolvidos nessa condição neuropsiquiátrica.

2.2 MICRORNA

Os microRNAs (miRNAs) são RNAs não codificantes de aproximadamente 21 a 25 nucleotídeos, que controlam inúmeras atividades desde desenvolvimento à morte celular. São estruturas de fitas simples que se ligam ao RNAm de forma parcial levando a regulação pós-transcricional da expressão gênica, podendo agir via degradação ou bloqueio da tradução (WAHID et al., 2010).

Historicamente, em 1993, o primeiro miRNA, lin-4, foi descoberto em *Caenorhabditis elegans*; o segundo, let-7, em 2000, ambos associados ao tempo de desenvolvimento do nematódeo. Depois desses achados, inúmeros relatos sobre as relações miRNA-RNAm e os resultados da regulação funcionais foram documentados (KAUR; ARMUGAM; JEYASEELAN, 2012). O conceito inicial de miRNAs como

reguladores do desenvolvimento tem sido expandido. Em situações patológicas, incluindo câncer, hepatite e doenças cardiovasculares, apresentam-se desregulados. Essa desregulação é devido a eventos genômicos, como mutações, deleções, inserções ou alterações transcricionais, ou defeitos na biogênese do miRNA devido a mutações ou a downregulação de enzimas envolvidas na formação do miRNA maduro (RUPAIMOOLE; SLACK, 2017).

MiRNAs são transcritos a partir de regiões intergênicas, embora uma minoria considerável, de regiões intrônicas. Aproximadamente 50% de miRNAs são encontrados próximos a outros miRNAs, indicativo de cluster de miRNAs serem transcritos a partir de uma única unidade de transcrição policistrônica (poligênica). Uma análise detalhada da expressão do gene mostraram que os genes de miRNAs podem ser transcrito a partir de seus próprios promotores, e que o miRNAs em cluster são gerados como transcritos primários policistrônico (pri-miRNAs) (KIM, 2005).

Os miRNAs de mamíferos são transcritos pela RNA polimerase II ou RNA polimerase III, gerando um miRNA primário (pri-miRNA), formado por uma ou mais estruturas em forma de grampo. Os pri-miRNAs são processados/clivados por um complexo microprocessador nuclear composto pela enzima Drosha (um tipo de RNase III), e um essencial cofator DGCR8. Nocautes em DGCR8 em camundongos e células-tronco embrionárias promovem, respectivamente, letalidade e defeitos na proliferação e diferenciação, mostrando a sua importância durante o desenvolvimento. O produto do microprocessamento nuclear é o pré-miRNA, formado por ~70 nucleotídeos. Alguns pri-miRNAs intrônicos, após o processo de splicing, apresentam um tamanho apropriado e uma conformação de grampo semelhante a um pré-miRNA. Nesse caso, ignoram a clivagem pela DROSHA e são levados diretamente ao citoplasma para continuar o processo. Após a formação do pré-miRNA, este é protegido e transportado ao citoplasma em associado ao complexo Exportin-5-RAN-GTP-dependente (SIOMI; SIOMI, 2010).

No citoplasma, o pré-miRNA é processado pelo complexo enzimático DICER/TRBP/PACT, perdendo a configuração em “grampo” e originando duas fitas simples de miRNA de aproximadamente 22 nucleotídeos (duplex). Os duplex de pequeno RNA gerados por DICER são posteriormente carregados sobre uma proteína argonauta (AGO) para formar um complexo efetor chamado complexo de silenciamento

induzido por RNA (RISC). Em humanos, os duplex de miRNA podem ser carregados por 4 tipos de AGO (AGO1-4). Após o carregamento do duplex miRNA, o miRNA maduro (5p) é retido no miRISC, enquanto que a fita complementar, conhecida como miRNA estrela - miR * (3p), é liberada. A fita guia (5p) é definida durante a etapa de carregamento da AGO, principalmente com base na estabilidade termodinâmica relativa entre as extremidades do pequeno RNA duplex, sendo a cadeia com terminal relativamente instável no lado 5', selecionado como a fita guia. Um determinante adicional da escolha é o primeiro nucleotídeo da sequência, pois as proteínas AGO selecionam como fita guia, a sequência de nucleotídeo que possuir um U na posição 1. O RISC, contendo o microRNA, liga-se aos mRNAs-alvo, geralmente na 3´-UTR, mas pode se ligar a qualquer outra região que apresente complementariedade, aumentando a diversidade de alvos de cada miRNA (COOLEN; KATZ; BALLY-CUIF, 2013)(WINTER et al., 2009)(KIM, 2005)(WAHID et al., 2010). A figura 4 ilustra a biogênese e atuação dos miRNAs descritos previamente.

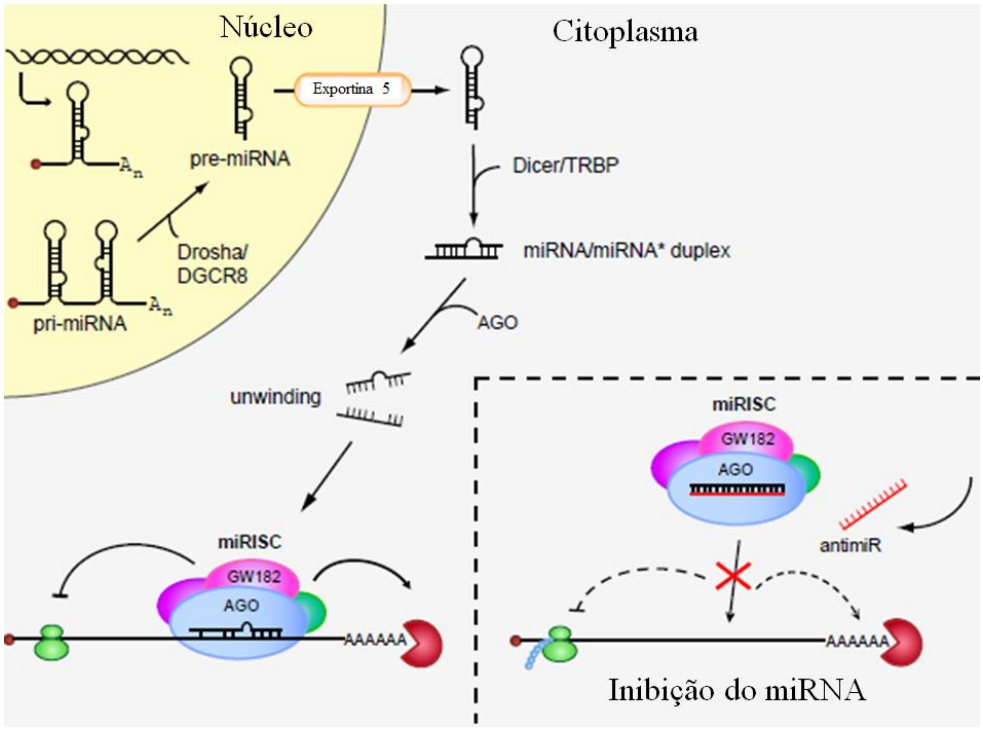


Figura 4 - Esquema da biogênese e atuação dos miRNAs. Os genes miRNA são transcritos por RNA Polimerase II em transcritos de miRNA primários (pri-miRNAs) com um CAP 5' e uma cauda poli (A). Pri-miRNAs são processados no núcleo para pré-miRNAs, composto por ~ 70 nucleotídeos, pelo complexo nuclear DGCR8 e RNase III enzima Drosha. Os pré-miRNAs são exportados para o

citoplasma pela Exportina-5 e processados pela DICER, para ~ 22 nucleotídeos miRNA de cadeia dupla - duplex que são carregados em uma proteína argonauta no miRISC. Durante este processo, o miRNA maduro é mantido em miRISC, enquanto a cadeia complementar, conhecida como a estrela miRNA (miR *) é liberada. MiRISC se liga parcialmente aos sítios complementares nos alvos 3 'UTRs dos mRNAs para promover repressão tracnslacional, deadenilação e degradação. AntimiR modificados sequestram o miRNA maduro em competição com mRNA alvo celular, levando a inibição funcional do miRNA e não repressão dos alvos diretos. Fonte: adaptado de Stenvang et. al, 2012.

MicroRNA é uma conhecida ferramenta de regulação gênica, envolvido na regulação de diversos processos biológicos (ZIMMERMAN; WU, 2011). Em mamíferos, mais de 1000 miRNAs foram identificados ou preditos *in silico*. Prevê-se que um único miRNA pode atingir centenas de alvos, por outro lado, a expressão de cerca de um terço de genes humanos é regulada por vários miRNAs, já sendo associados a regulação da proliferação, apoptose, resposta ao stress, desenvolvimento embrionário, ciclo celular, a iniciação e progressão do tumor, metástase e diferenciação de células-tronco (WANG; BLELLOCH, 2010).

Identificação e validação experimental é um pré-requisito essencial para descobrir papéis biológicos mediados por miRNAs e integração de redes reguladoras de genes. Isso acelerou o desenvolvimento de vários recursos computacionais, bioquímicos, abordagens genéticas e funcionais para miRNA (M. WITKOS; KOSCIANSKA; J. KRZYZOSIAK, 2011). Atualmente, três abordagens são usadas no estudo de perda função de miRNA: nocautes genéticos, miRNAs esponjas e oligonucleotídeos. Uma abordagem amplamente empregada é o uso de oligonucleotídeos anti-sense modificados quimicamente, denominados antimicroRNAs (antimiR), que sequestram o miRNA maduro em competição com alvo celular, conduzindo à inibição funcional do miRNA e desregulação dos alvos (EBERT; NEILSON; SHARP, 2013). A inibição de miRNA por antimiRs requer otimização de oligonucleotídeos para melhorar a afinidade de ligação, resistência melhorada à nuclease e entrega *in vivo*. Esse melhoramento pode ser alcançado usando uma variedade de modificações químicas, incluindo modificações do açúcar, nas bases nucleotídicas ou nas ligações internucleotídicas (STENVANG et al., 2012).

O miRNA tem sido amplamente utilizado em estudos funcionais de genes, em uma variedade de campos, como uma nova ferramenta na regulação da expressão

gênica. Como uma tecnologia emergente, tem potencial para ser empregado no desenvolvimento farmacológico. Os avanços recentes na pesquisa forneceram mais informações sobre a biogênese, função e sítios de ligação do miRNA. Sendo assim, os miRNAs identificados e associados a patologias são promissores alvos terapêuticos.

2.2.1 *MicroRNA-9*

O microRNA-9 (miR-9) vem sendo amplamente estudado por apresentar alta e específica expressão no sistema nervoso central (SNC) e possuir sequência altamente conservada em metazoa (COOLEN; KATZ; BALLY-CUIF, 2013). Ao passo que sua função e seu espectro de ação começaram a ser desvendados, o miR-9 revelou-se altamente versátil, exercendo várias atividades, às vezes antagônicas, dependendo do contexto celular e das espécies.

MiR-9 tem a habilidade de regular o crescimento, a diferenciação, a migração, e apoptose de células cancerosas, seja como oncogênese ou como supressor tumoral dependendo do tipo de câncer (CHEN et al., 2015). MiR-9 maduro vem dos três transcritos primários pri-miR-9-1, pri-miR-9-2 e pri-miR-9-3, localizados nas regiões cromossômicas 1q22, 5q14.3 e 15q26.1, respectivamente, podendo originar miR-9-5p (miR-9) e miR-9-3p (miR-9*) (Figura 5). A fita complementar (miR-9*) é menos estudada e tem menor taxa de expressão (NOWEK et al., 2016). A expressão de miR-9* está correlacionada ao desenvolvimento neural e à modulação do crescimento dendrítico (STAPPERT; ROESE-KOERNER; BRÜSTLE, 2015). A associação de miR-9 e miR-9* (miR-9/miR9*) pode potencializar a regulação de um alvo ou atuar de forma antagônica. Esse antagonismo foi observado na relação miR-9/ cMYC , onde miR-9 é up-regulado por c-MYC, (ZAWISTOWSKI et al., 2013), cujo papel está relacionado com progressão do ciclo celular, apoptose e diferenciação (DANG et al., 1999), e o aumento da expressão do miR9* levou a redução da expressão da proteína c-MYC. Por outro lado, a simultânea ação do MiR-9/ MiR9* regula a autorrenovação até a diferenciação neuronal em células-tronco neuroepiteliais (células lt-NES) via NOTCH (ROESE-KOERNER et al., 2016).

Em células progenitoras neurais adultas (NPCs), miR-9 regula a proliferação celular e acelera as diferenciações neurais, enquanto que nos primeiros estágios do

NPCs provenientes de células-tronco humana ,estágio de neurosfera, o miR-9 aumenta a proliferação e suprime a migração de NPCs (TOPOL et al., 2016). De modo geral, os estudos mostram que o miR-9 possui funções dependentes do estágios do desenvolvimento do cérebro, da região cerebral, da espécie e tipo celular , podendo promover ou suprimir a proliferação e diferenciação de NPCs (COOLEN; KATZ; BALLY-CUIF, 2013). Informações sobre o papel do miR-9 em outros tecidos não são muito comuns, embora tenha sido detectado durante diferenciação dos hepatócitos, desenvolvimento da retina e associado a regulação de respostas inflamatórias (LUKIW et al., 2012).

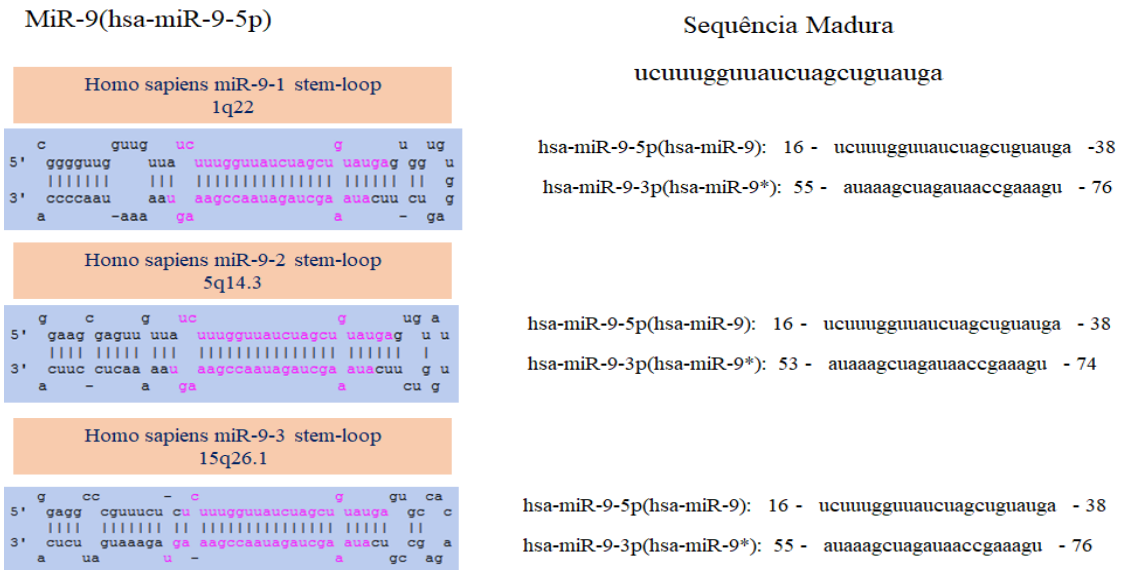


Figura 5 - Sequência miR-9. O miR-9 pode vir de diferentes loci gênicos. A figura acima mostra a sequência de origens, dos três transcritos primários pri-miR-9-1, pri-miR-9-2 e pri-miR-9-3, localizados em 1q22, 5q14.3 e 15q26.1, respectivamente, dando a origem a duas sequências reguladoras miR-9 e miR-9*. Informações obtidas de http://www.mirbase.org/. Fonte: Produção do próprio autor.

No câncer de fígado, o miR-9 atua potencialmente como um oncogênese, regulando a iniciação do tumor, crescimento e metástase, apresentando um nível de expressão proporcional à gravidade e extensão do tumor. A inibição da expressão de miR-9 (anti-miR-9) bloqueia as propriedades tumorais de células de câncer de fígado, incluindo crescimento celular e migração, sugerindo ser um potencial alvo terapêutico (DRAKAKI et al., 2015).

MiR-9 foi identificado como uma espécie indutora de PDGFB que pode interagir diretamente com o sítio 3'UTR do PDGFRB, podendo regular o PDGFRB quando os níveis intracelulares estão aumentados, o que resultou em uma diminuição da capacidade angiogênica em cardiomiócitos. Além disso, o bloqueio induzido por antagonista do miR-9 atenuou uma diminuição da regulação de PDGFRB em cardiomiócitos, fornecendo forte evidência de regulação de PDGFRB envolvendo miR-9 (ZHANG et al., 2011). O fator de crescimento derivado das plaquetas-BB (PDGFB) tem um aumento da regulação mediada por miR-9, que regula negativamente a proliferação em monócitos, diferenciação neuronal, bem como migração de células progenitoras neuronais (NPCs) (YANG et al., 2013). Estudos *in silico*, mostrou o miR-9 como regulador do PDGFRB e SLC20A2. Essa regulação foi confirmada *in vitro* através de células HEK293, mostrando a capacidade supressora de genes ligados a calcificação cerebral primária familiar via miR-9 (PAIVA; KEASEY; OLIVEIRA, 2017).

A diversidade de alvos e modo de atuação do miR-9 torna-o uma importante ferramenta e alvo biológico no estudo de diversos tipos de câncer. Além de tecidos tumorais, o miR-9 tem predileção por tecido neural, fato que pode ser confirmado devido a habilidade de regular dois dos principais genes associados com calcificação cerebral.

Os estudos em pacientes com calcificação cerebral são bastante invasivos o que implica em limitações éticas, por conta disso, para entender melhor essa via de regulação, é necessária a criação de modelos de estudos para entender melhor a calcificação cerebral primária.

2.3 SAOS-2 / HEK293 – MODELOS CELULARES IN VITRO

Modelos *in vitro* geralmente são mencionados como a manipulação de tecidos, células e biomoléculas em um ambiente artificial e controlado. Vários modelos de cultura celular são empregados para tentar mimetizar condições e torná-las mais próximas das características *in vivo*. Modelos *in vitro* contornam conflitos de ordens éticas como estudos *in vivo*, envolvendo manipulação de animais, abortos e estudo em

seres humanos. Cultura celular é uma atrativa ferramenta para estudo de sistemas devido ter inúmeras linhagens celulares conhecidas e bem caracterizadas, podendo ainda algumas células serem obtidas por cultura primária.

Cultura primária é um processo de cultivo celular artificial, cuja fonte são células ou tecidos isolados por desagregação mecânica, desagregação enzimática e explante. As células são mantidas e propagadas (repicadas) *in vitro* em meio nutritivo adequado ao tipo celular. São células com perfis heterogêneos devido a características individuais como fatores genéticos e idade do indivíduo, que podem alterar a caracterização e respostas celular . Embora as células primárias ofereçam vantagens, a obtenção de uma população uniforme pode ser um processo complexo e adverso, pois são sensíveis, exigem suplementação de nutrientes não inclusos nos meios clássicos. Para otimizar a sobrevivência e o crescimento, as células primárias funcionam melhor em meios especiais personalizados para cada tipo celular. Linhagens celulares são células geneticamente manipuladas tendo como origem as linhagens primárias, devendo exibir e manter propriedades mais próximas das originais. São fáceis de manusear e fornecem uma população pura de células. As linhagens celulares podem alterar seu fenótipo, genótipo, funções e respostas durante sucessivas passagens, podendo gerar uma cultura heterogênea a longo prazo (FERREIRA; ADEGA; CHAVES, 2013; MACK et al., 2013)

Entre as linhagens mais comuns estão as células HEK-293 (Human Embryonic Kidney 293 cells) com propriedades de crescimento e transfecção que as tornam atraentes ferramentas para as indústrias biofarmacêuticas. As células HEK293 são úteis para muitas experiências de transfecção, particularmente a propagação de vectores baseados em adenovírus e retrovirais. São facilmente cultivadas em meio de cultura sem soro, tem alto índice de crescimento e transfecção, e são altamente eficientes em produção de proteínas (DUMONT et al., 2016).

As células SaOs-2 (Osteosarcoma Cell Line) são uma linhagem imortalizada de osteosarcoma humano com propriedades de osteoblasto, cuja principal característica é a presença de matriz mineralizada, associada com elevada fosfatase alcalina (RODAN et al., 1987). Além disso, estudos citoquímicos e moleculares mostraram a expressaram de marcadores osteoblásticos, sialoproteína óssea, decorina; e pro-colágeno-I, outras proteínas como colágeno tipo III e osteoprotegerina (OPG), que só foram detectadas,

aproximadamente, em 15% das células (PAUTKE et al., 2004). Essa predisposição a diferenciação osteogênica, devido a propriedade inatas e adquiridas durante o processo de imortalização, faz das células SaOs-2 um interessante modelo para estudo de calcificação.

A habilidade de diferenciação, faz das células SaOs-2 um controle positivo para estudo de calcificação para outros tipos celulares e protocolos experimentais (MORI et al., 1999). Keasey et al., mostrou que a partir de indução química com ácido ascórbico e β-glicerolfosfato as células SaOs-2, após 14 dias de diferenciação, adquiriram um fenótipo ósseo, caracterizado por deposição de hidroxiapatita, confirmada pela coloração alizarin red. O aumento da calcificação foi associado a diminuição da expressão do PiT2 (KEASEY et al., 2016).

Grande parte do conhecimento atual sobre doenças neurológicas foi adquirida a partir de estudos pós-morte devido às limitações para obtenção de tecido cerebral vivo. Isto torna problemática a compreensão do desenvolvimento e progressão da doença, pois amostras pós-morte só representam o estágio final da doença, e os aspectos da patologia observadas nestas amostras pode ser secundária e não fielmente refletir o fenótipo da doença exata a um nível celular. Diferenças entre espécies dificultam a simulação de doenças neurológicas humanas em modelos animais. Portanto, a modelagem de doença simulando o fenótipo da doença *in vitro* e em populações celulares é um importante avanço e tornaria possível compreender os mecanismos celulares e moleculares de desordens neurodegenerativas.

Estudos mais recentes começaram a também a caracterizar aspectos da eletrofisiologia da SaOs-2, investigando também a caracterização de canais iônicos e resposta à ação de alguns inibidores de potencial de membrana (Seabra et al, 2016). http://www.cell.com/biophysj/abstract/S0006-3495(15)03576-6

Ainda há muito a ser estudado em relação ao fenômeno das calcificações em partes moles (vasos, olhos, articulações, mamas, válvulas cardíacas, etc). Por isso, é fundamental se explorar modelos e métodos que possam replicar, *in vitro*, processos patológicos vistos em pacientes. Isso vai permitir um melhor entendimento das bases moleculares das CCPF e também facilitará a identificação de novas metodologias diagnósticas e terapêuticas.

Nossa abordagem se limitou a alguns modelos que pareceram replicar satisfatoriamente parte dos achados visto em pacientes, principalmente com o uso de métodos de indução química

3 Objetivos

Objetivo Geral

- Observar a influência do miR-9-5p no desenvolvimento ou aprimoramento da calcificação *in vitro*, focado nos genes ligados à CCPF.

Objetivos específicos

- Criar modelos *in vitro* para estudo das calcificações cerebrais familiar primárias ligadas aos genes *SLC20A2* e *PDGFRB*.
- Estudar Calcificações cerebrais familiar primárias (CCPF) em SaOS-2, *in vitro*.
- Observar a presença do microRNA-9 no desenvolvimento ou na potencialização de calcificações, *in vitro*.

4 ARTIGO 1 : NEW STUDIES ON KNOCKOUT MOUSE FOR THE SLC20A2 GENE SHOW MUCH MORE THAN BRAIN CALCIFICATIONS, PUBLICADO NA JOURNAL MOLECULAR NEUROSCIENCE

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New Studies on Knockout Mouse for the *SLC20A2* Gene Show Much More Than Brain Calcifications

D. P. Bezerra[1,2] · J. R. M. Oliveira[1,2,3]

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To the editor

We read with great interest the recent contribution by Jensen et al. (2015) and Wallingford et al. (2016a, b), reporting additional phenotyping data for *SLC20A2* knockout (KO) mouse, originally described in 2013 (Jensen et al. 2013). The *SLC20A2* KO was evaluated as a model for human primary familial brain calcification (PFBC) and appears to correlate well with the loss of function mutants found in PFBC patients, where SLC20A2 represents ~40-50 % of inherited cases (Femia et al. 2014). The reported similarities between *SLC20A2* KO mouse and human pathologies was striking.

Now, Jensen et al. (2015) show significantly elevated inorganic phosphate levels (Pi) in cerebrospinal fluid (CSF) from the *SLC20A2* KO, confirming PIT-2 regulates Pi in the CSF. Separately, Wallingford et al. (2016a, b) described several additional central and peripheral phenotypes in *SLC20A2* −/− mouse, including: elevated phosphate in CSF and CSF producing tissues (choroid plexus, ependymal, arteriolar smooth muscle cells), calcified optic nerve tissue, and hydrocephalus. Both studies confirm the importance of *SLC20A2* in Pi homeostasis and clearance from the CSF generating tissues, increasing the vulnerability of smooth muscle cells to glymphatic pathway-associated arteriolar calcification. They also report that heterozygous mice would present calcifications, as they get older, mimicking the patient profile.

PFBC is characterized by symmetric calcification in the basal ganglia and other brain regions, including a wide spectrum of neuropsychiatric symptoms such as parkinsonism, psychosis, seizures, and chronic headache. The usage of "primary" alludes to the lack of hormonal, infectious, or traumatic causes that could lead to brain calcifications (Sobrido et al. 2014). PFBC is inherited in an autosomal dominant manner and, thus far, four genes have been identified. Two have been linked to phosphate metabolism (*SLC20A2*, *XPR1*), while the other two were initially associated with blood brain barrier (BBB) integrity and pericytes maintenance (*PDGFB*, *PDGFRB*)(Tadic et al. 2015).

The Wallingford et al. (2016a, b) analysis challenges previous interpretations linking brain calcification to BBB integrity, suggesting that brain calcifications are found even with normal BBB functioning, through a two-hit mechanism whereby increased CSF Pi leads to calcification in arteriolar smooth muscle cells due to an enhanced vulnerability caused by *SLC20A2* deficiency. This idea is further supported by the recent questioning of the BBB hypothesis as a potential cause for PFBC. Part of the original group who linked PDFGB BBB deficiency with calcification in *PDGFB* KO mice found that calcification-prone brain regions had a more intact blood–brain barrier (BBB) and higher pericyte coverage compared to non-calcification-prone brain regions (Vanlandewijck et al. 2015). On the other hand, a patient reported with brain calcification, and a novel *PDGFB* mutation presented with electron microscopy of skin biopsy showing capillary basal membrane abnormalities consistent with microangiopathy (Blanchet et al. 2016).

Large scale phenotyping of this very same *SLC20A2* KO is available in public repositories (http://www.mousephenotype.

✉ J. R. M. Oliveira
jmoliveira@ufpe.br

[1] Keizo Asami Laboratory-Federal University of Pernambuco (UFPE), Recife, PE, Brazil

[2] Biological Sciences Graduate Program, UFPE, Recife, PE, Brazil

[3] Neuropsychiatry Department, UFPE, Recife, PE, Brazil

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Table 1 SLC20A2 phenotyping data retrieved from WWW.MOUSEPHENOTYPE.ORG/

Strain: $SLC20A2^{tm1a(EUCOMM)Wtsi}$ (HOMOZYGOTE)	• ABNORMAL LENS AND IRIS MORPHOLOGY WITH CATARACT • ABNORMAL BONE STRUCTURE AND SPINE CURVATURE, INCLUDING KYPHOSIS; • DECREASED BODY LENGTH; • DECREASED BONE MINERAL DENSITY AND CONTENT • DECREASED FASTED CIRCULATING GLUCOSE LEVEL; • DECREASED LEAN BODY MASS AND TOTAL BODY FAT • INCREASED CIRCULATING ALKALINE PHOSPHATASE LEVEL; • INCREASED CIRCULATING MAGNESIUM LEVEL

org) (see Table 1), showing changes in skeleton, eyes, and general growth impairment, something that was not properly checked in patients (Brown and Moore 2012). This might be explained by the fact that the homozygous knockout has a more deleterious phenotype. However, repository data is often based on a comparison of historical controls and usually using a smaller sample size, in a high throughput setup. In this case, the characterization might vary when compared with a more controlled study.

Making a clinical diagnosis of PFBC relies on the combination of clinical features, brain imaging, and exclusion of other causes of intracranial calcification. Normal serum levels of calcium, phosphorus, alkaline phosphatase, and parathormone can help in differentiating it from endocrine disorders (Sobrido et al. 2014). Although invasive, the CSF level of Pi may be a new parameter used to investigate suspect cases of PFBC.

More recently, Wallingford et al. (2016b) found that this same model presents lower fetal viability, most likely due to abnormal placental function. This highlights that such models may help to elucidate multiple human conditions linked to inorganic phosphate homeostasis.

It is encouraging, for researchers and patients, to see a recent rise in interest in an animal model for a disease with so few alternatives for treatment that slow or stop disease progression.

Preliminary data presented by one of the groups responsible for discovering the link between *SLC20A2* and PFBC reports that another KO mouse, now homozygous for a specific mutation, found initially in Chinese patients (S602W), also replicates the PFBC findings in the brain. Without further details, they claim that a Chinese herbal medicine formula was able to produce preventive effects in brain calcification formation (Wang et al. 2015).

More recently, our group reported seven patients with brain calcification treated with biphosphonate alendronate, arguing for a thorough evaluation of bisphosphonates as a preventive treatment for such a devastating condition still underdiagnosed and with few therapeutic options (Oliveira and Oliveira 2016).

References

Biancheri R, Severino M, Robbiano A et al (2016) White matter involvement in a family with a novel *PDGFB* mutation. Neurol Genet 2, e77. doi:10.1212/NXG.0000000000000077

Brown SDM, Moore MW (2012) The International Mouse Phenotyping Consortium: Past and future perspectives on mouse phenotyping. Mamm Genome 23:632–640. doi:10.1007/s00335-012-9427-x

Ferreira JB, Pimentel L, Keasey MP et al (2014) First Report of a De Novo Mutation at SLC20A2 in a Patient with Brain Calcification. J Mol Neurosci 54:748–751. doi:10.1007/s12031-014-0357-9

Jensen N, Schrader HD, Hejbøl EK et al (2013) Loss of function of Slc20a2 associated with familial idiopathic Basal Ganglia calcification in humans causes brain calcifications in mice. J Mol Neurosci 51:994–999. doi:10.1007/s12031-013-0085-6

Jensen N, Autzen JK, Pedersen L (2015) Slc20a2 is critical for maintaining a physiologic inorganic phosphate level in cerebrospinal fluid. Neurogenetics 17:125–130. doi:10.1007/s10048-015-0469-6

Oliveira JRM, Oliveira MF (2016) Primary brain calcification in patients undergoing treatment with the biphosphonate alendronate. Sci Rep 6:22961

Sobrido MJ, Coppola G, Oliveira J, Hopfer S, Geschwind DH (2014) Primary Familial Brain Calcification. In: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Fong CT, Mefford HC, Smith RJH, Stephens K, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle

Tadic V, Westenberger A, Domingo A et al (2015) Primary Familial Brain Calcification With Known Gene Mutations. JAMA Neurol 72:460. doi:10.1001/jamaneurol.2014.3889

Vanlandewijck M, Lebouvier T, Andaloussi Mäe M et al (2015) Functional Characterization of Germline Mutations in PDGFB and PDGFRB in Primary Familial Brain Calcification. PLoS One 10, e0143407. doi:10.1371/journal.pone.0143407

Wallingford M, Chia J, Leaf E, et al. (2016a) SLC20A2 deficiency in mice leads to elevated phosphate levels in cerebrospinal fluid and glymphatic pathway-associated arteriolar calcification, and recapitulates human idiopathic basal ganglia calcification. Brain Pathol 3: [Epub ahead of print]. doi: 10.1111/bpa.12362

Wallingford MC, Gammill HS, Giachelli CM (2016b) Slc20a2 deficiency results in fetal growth restriction and placental calcification associated with thickened basement membranes and novel CD13 and laminin1 expressing cells. Reprod Biol 16:13–26. doi:10.1016/j.repbio.2015.12.004

Wang C, Xu X, Luo J, Liu J (2015) Chinese herbal medicine formula SYM prevents brain calcification in a murine model of IBGC. Chin J Pharmacol Toxicol 29(Suppl 1):41–42

5 ARTIGO 2 : MIR-9-5P DOWN-REGULATES PIT2, BUT NOT PIT1 IN HUMAN EMBRYONIC KIDNEY 293 CELLS, PUBLICADO NA JOURNAL MOLECULAR NEUROSCIENCE

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MiR-9-5p Down-Regulates PiT2, but not PiT1 in Human Embryonic Kidney 293 Cells

D.P. Paiva[1] · M. Krassy[1,2] · J. R. M. Oliveira[1,3]

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Abstract Inorganic phosphate (Pi) is an essential component for structure and metabolism. PiT1 (*SLC20A1*) and PiT2 (*SLC20A2*) are members of the mammalian type-III inorganic phosphate transporters. *SLC20A2* missense variants are associated with primary brain calcification. MicroRNAs (miRNAs) are endogenous noncoding regulatory RNAs, which play important roles in post-transcriptional gene regulation. MicroRNA-9 (miR-9) acts at different stages of neurogenesis, is deeply rooted in gene networks controlling the regulation of neural progenitor proliferation, and is also linked with cancers outside the nervous system. We evaluated possible interactions between miR-9 and the phosphate transporters (PiT1 and PiT2). SLC20A2, platelet-derived growth factor receptor beta (PDGFRB) and Fibrillin-2 (FBN2) showed binding sites with high affinity for miR-9, *in silico*. miR-9 mimic was transfected into HEK293 cells and expression confirmed by RT-qPCR. Overexpression of miR-9 in these cells caused a significant reduction in PiT2 and FBN2. PDGFRB appeared to be decreased, but was not significantly down-regulated in our hands. PiT1 showed no significant difference relative to controls. The down-regulation of PiT2 protein by miR-9 was confirmed by western blotting. In conclusion, we showed miR-9 can down-regulate PiT2, in HEK293 cells.

Keywords PiT2 · miR-9 · HEK293 cells

✉ D.P. Paiva
darlenepaiva@gmail.com

✉ M. Krassy
krassy@etsu.edu

✉ J. R. M. Oliveira
joao.ricardo@ufpe.br

[1] Keizo Asami Laboratory, Federal University of Pernambuco, Recife, PE, Brazil

[2] Neuropsychiatry Department, Federal University of Pernambuco, Recife, PE, Brazil

[3] Department of Biomedical Sciences – Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA

Introduction

Inorganic phosphate (Pi) is an essential component for structure and metabolism. Maintenance of Pi homeostasis involves a network of hormones, growth factors, and cell types (Sapio and Naviglio 2015). Type III Pi transporters are critical for inorganic phosphate transport in various tissues, including PiT1 and PiT2 as members of the mammalian type-III inorganic phosphate transporters encoded by the SLC20 genes (Bøttger and Pedersen 2002, 2011). Both genes are ubiquitously expressed in the brain (Inden et al. 2013) and are considered housekeeping genes, acting to maintain Pi homeostasis (Bøttger et al. 2006; Jensen et al. 2016).

Jensen et al. (2013, 2016) reported an *Slc20a2* Knockout (KO) mouse with significantly elevated inorganic phosphate levels in cerebrospinal fluid (CSF), reinforcing a role for SLC20A2 in Pi maintenance. *In vivo*, homozygous *Slc20a2* KO mice exhibit brain calcification as observed in primary familial brain calcification (PFBC) patients in the clinic. Strikingly, *Slc20a2* KO mice were also viable (Jensen et al. 2013, 2016).

These findings were supported by Wallingford et al. 2016, where high phosphate in the CSF occurred in the absence of changes in serum phosphate and calcium levels in *Slc20a2* heterozygous (+/−) or *Slc20a2* KO (−/−) mice. *Slc20a2* KO mice also displayed calcified optic nerve tissue, and moderate to severe hydrocephalus while Knockdown of *SLC20A2* in

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smooth muscle cells (SMCs) leads to enhanced susceptibility to calcification (Wallingford et al. 2016).

There is growing interest in SLC20A2 since the initial report of mutations linked to brain calcification in humans. PFBC is a rare disorder characterized by bilateral calcium phosphate deposition in different parts of the brain, associated with movement disorders, psychosis, dementia, and headache. Causative mutations have been found in SLC20A2 (Wang et al. 2012), XPR1 (Xenotropic and Polytropic Retrovirus Receptor 1) (Legati et al. 2015), PDGFRB (platelet-derived growth factor receptor β - PDGFRβ) (Nicolas et al. 2013), and in PDGFB (platelet-derived growth factor β) (Keller et al. 2013). However, SLC20A2 is responsible for most of the PFBC cases (Lemos et al. 2015). Despite this, the regulation of this gene is poorly understood.

The discovery of non-coding microRNAs (miRNAs) has shown a new layer of post-transcriptional gene regulation. The manipulation of miRNA expression levels and patterns in the nervous system has demonstrated the critical role they play in neurogenesis (Zhang et al. 2012) and cancers such as leukemias, thyroid carcinomas, breast, lung, and pancreatic cancer (Alexiou et al. 2009). Amongst various others, microRNA-9 (miR-9) is highly conserved and commonly found in intronic and intergenic regions throughout the genome, presenting three mature forms: *MIR9-1* (1q22), *MIR9-2* (5q14.3), and *MIR9-3* (15q26.1) (Chen et al. 2015). The guide strand of miR-9 can be generated either from the 5′ (miR-9-5p) or the 3′ arm (miR-9-3p) (Coolen et al. 2013). miR-9 is implicated in functions in the central nervous system (CNS): expansion and differentiation, axonal extension, and branching and axon guidance (Cao et al. 2016). miR-9 is also a known regulator of PDGFRβ expression in cardiomyocytes (Zhang et al. 2011).

In this work, we set out to identify possible interactions between miR-9-5p and the phosphate transporter SLC20A2.

Methods

Bioinformatics prediction

The binding affinity of the miR-9-5p to PiT2 was predicted using the online algorithms "TargetScan" and DianaTools, which are available online at http://www.targetscan.org/docs/UTR_profiles.html and http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index, respectively. These programs were used due to their higher selectivity and fewer false positive targets (Alexiou et al. 2009).

Cell culture

HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Cat # 12100046, Gibco) supplemented with 10% fetal bovine serum (SFB) (Cat # 10437028, Gibco), 1 mM glutamine (Cat # 25030081, Gibco), and 10,000 U/mL penicillin and streptomycin (Cat# 15140122 Invitrogen, USA), kept at 37 °C with 5% CO2 with approximately 95% humidity. Cell splitting happened every 3 days.

Transfections

Cells were seeded onto 6-well plates (1×10^5 cells per mL). After 24 h (50% confluent), the medium was removed and stored for later use (conditioned media). HEK293 cells were transfected with miR-9-5p (1, 3, 10, and 30 nM, Cat# PM10022, Ambion) or Cy3 (30 nM, Cat# AM17120, Ambion). All transfections were carried out in triplicate with Lipofectamine 2000 (Cat# 11668027, Invitrogen) and Opti-MEM medium (Cat# 31985070, Gibco). After 4 h, medium was removed and replaced with the conditioned media. RNA and Protein were extracted after 24 h.

Western blot

Protein isolation was performed according to Keasey et al. (Keasey et al. 2013). Antibodies used were PiT2 (sc-377326 - 1:1000, Santa Cruz, CA, USA) and GAPDH (sc-25778, 1:1000, Santa Cruz). Signal was developed with a HRP conjugated anti-rabbit (sc-2030) and anti-mouse (sc-2005) secondary antibody and ECL substrate (Cat# WP20005, Invitrogen). PiT2 protein levels were quantified by densitometry using the ImageJ system, and expressed as ratio relative to GAPDH.

Real time qPCR (qRT-PCR)

RNA isolation, cDNA and qPCR were performed according to Keasey et al. (2016). With 0.5 μL of 20× TaqMan Gene Expression Assay (GAPDH, #Hs02758991_g1; SLC20A2, #Hs00198840_m1; SLC20A1, #Hs00965587_m1; FBN2 #Hs00266592_m1;PDGFRB, #Hs00387364_m1; miR-9-5p #PN4427975; U6 #PN4427975_m1 from Applied Biosystems). The normalized relative transcription levels were calculated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistics

Data were analyzed using GraphPad Prism (Version 5a) software using a one-way Anova with a Dunnett's post-hoc test ($p < *0.05$ and $**0.001$).

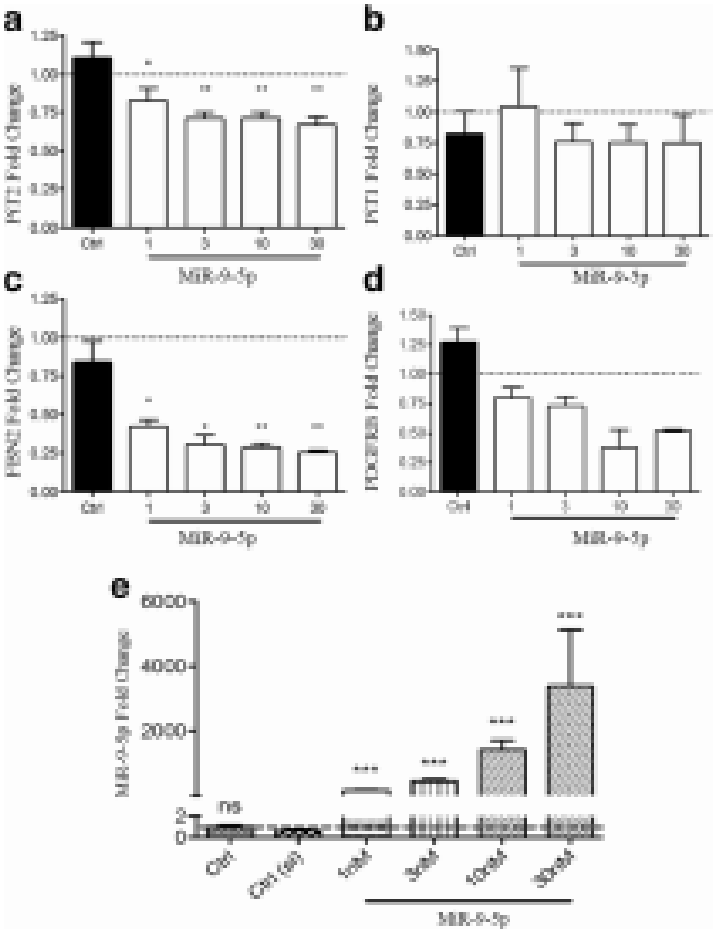


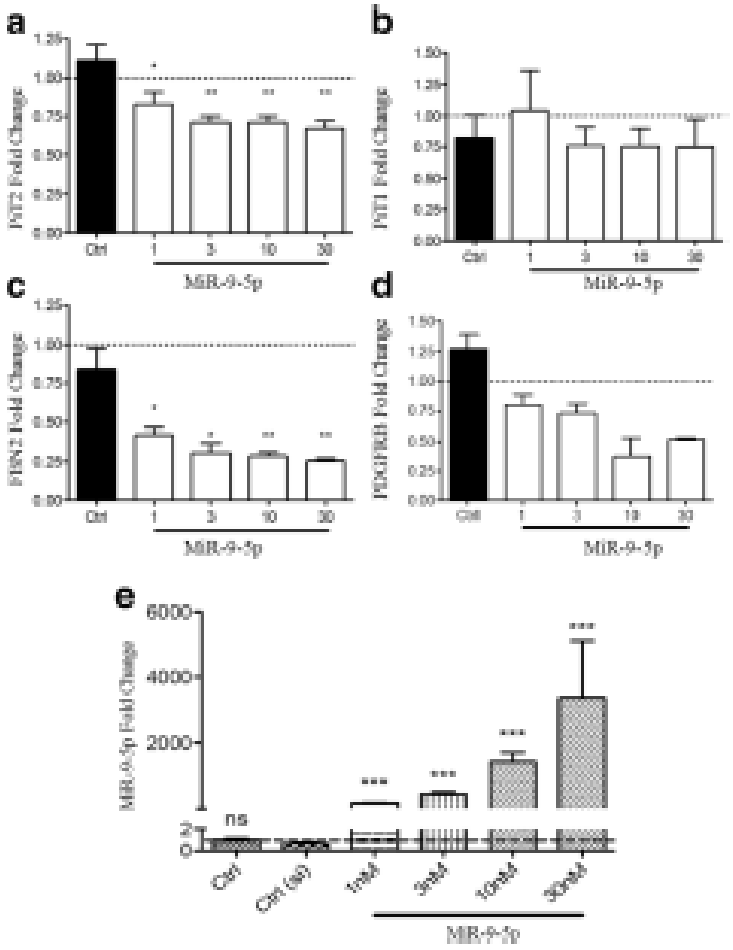
Fig. 2 In vitro, miR-9-5p transfection reduces expression of Pi transporter SLC20A2, in HEK293 cells. HEK293 cells were transfected with miR-9-5p. **a** SLC20A2 was slightly but significantly reduced in miR-9-5p transfected cells while SLC20A1 (**b**) was unchanged. **c** Fibrillin-2 was included as a positive control and was significantly decreased by miR-9-5p. **d** PDGFRB also appeared to be decreased but the changes shown were not significant in our hands. **e** miR-9-5p expression was confirmed by qPCR compared with controls (non-targeting cy3 conjugated siRNA transfected cells). *Black bars* represent Control (Ctrl) conditions and all are representative of means from 3 independent experiments ± SEM with one-way ANOVA ($p < *0.05$ and $**0.000$)

Discussion

This is the first report of SLC20A2, a key mediator of brain calcification both in the clinic and the laboratory, being regulated by a miRNA. Two recent studies showed that mice with slc20a2 KO and haploinsufficiency presented with high phosphate levels in CSF, potentially involved in development of vascular brain calcification (Jensen et al. 2016; Wallingford et al. 2016). Here we confirmed bioinformatics predictions and open a venue for the SLC20A2 gene modulation.

SLC20A2 mutations are responsible for the majority of reported PFBC cases. PFBC is a heterogeneous neuropsychiatric disorder with bilateral calcifications in the basal ganglia, thalamus, and cerebellum. Others genes have also been linked to PFBC: PDGFRB, PDGFB, and XPR1.

Keasey et al. (2016) reported that SLC20A2 expression is maintained by vitamin D in vitro whilst reducing calcification (hydroxyapatite deposition). SLC20A2 knockout ablated the vitamin D rescue effect, demonstrating that vitamin D could reduce calcification specifically by upregulating SLC20A2 (Keasey et al. 2016). PFBC patients with PiT2 mutants and animal models with knockout PiT2 demonstrate brain calcification, suggesting that PiT2 is an important protein involved in mineralization. Although our understanding of the causes of brain calcification has taken several steps forward in recent years, with the identification of several genes associated with or directly causing mineralization in one model or another. The specific cell type associated with this calcification remains to be determined. It is intriguing though, that PDGFRB, an important factor in pericyte recruitment to the blood–brain barrier is one of these identified genes, perhaps suggesting that leakiness of the vasculature may be a culprit (Nikanorova et al. 2010). Indeed, deposits seem to localize around the microvasculature in the brain (Mocarney and Squier 2014). In our hands, SLC20A2 was significantly suppressed while PDGFRB appeared to be decreased in response



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SLC20A2 mutations are responsible for the majority of reported PFBC cases. PFBC is a heterogeneous neuropsychiatric disorder with bilateral calcifications in the basal ganglia, thalamus, and cerebellum. Others genes have also been linked to PFBC: PDGFRB, PDGFB, and XPR1.

Keasey et al. (2016) reported that SLC20A2 expression is maintained by vitamin D in vitro whilst reducing calcification (hydroxyapatite deposition). SLC20A2 knockout ablated the vitamin D rescue effect, demonstrating that vitamin D could reduce calcification specifically by upregulating SLC20A2 (Keasey et al. 2016). PFBC patients with PiT2 mutants and animal models with knockout PiT2 demonstrate brain calcification, suggesting that PiT2 is an important protein involved in mineralization. Although our understanding of the causes of brain calcification has taken several steps forward in recent years, with the identification of several genes associated with or directly causing mineralization in one model or another. The specific cell type associated with this calcification remains to be determined. It is intriguing though, that PDGFRB, an important factor in pericyte recruitment to the blood–brain barrier is one of those identified genes, perhaps suggesting that leakiness of the vasculature may be a culprit (Nicolas-Jilwan et al. 2010). Indeed, deposits seem to localize around the microvasculature in the brain (Mecawney and Squier 2014). In our hands, SLC20A2 was significantly suppressed while PDGFRB appeared to be decreased in response

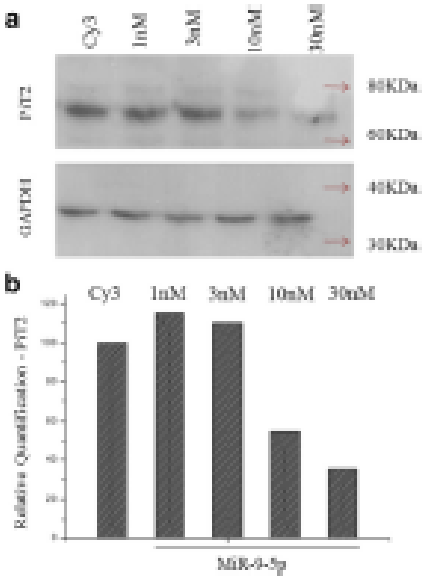

Fig. 3 miR-9-5p suppressed SLC20A2 protein expression in vitro. a Western blot analysis confirmed that protein expression (PiT2) was reduced in miR-9-5p transfected cells (representative of $n = 2$). b miR-9-5p overexpression reduced PiT2 protein by 54.8% (10 nM) and 35.2% (30 nM) relative the housekeeping GAPDH, quantified in the ImageJ software

to miR-9-5p overexpression. The possibility that miR-9-5p may regulate 2 genes associated with brain calcification may suggest it is a potent regulator of this pathology.

PiT2 is a member of the Pi transport (PiT) family (SLC20), which were originally identified as receptors for retroviruses belonging to the gammaretrovirus genus and constitute the housekeeping Pi uptake system between cells and the extracellular fluid. They show a broad tissue distribution, since both are expressed in all investigated human tissues, in different levels, although in mammalian cells low extracellular Pi levels can result in upregulated PiT1 and PiT2 expression (Kavanaugh et al. 1994). We demonstrated that HEK 293 cells, after miR-9-5p overexpression, showed a significant decrease in PiT2 expression but not PiT1 which fits with our *in silico* analysis, where we found a potential binding site for miR-9-5p within SLC20A2.

MicroRNAs (miRNAs) have been demonstrated to play vital roles in tumorigenesis and tumor progression. The majority of the effects of miRNAs were mediated through targeting different genes by regulating their expressions. Aberrant expression of microRNAs becoming evident in multiple disorders or in different types of cancer, pathogenic human viruses (Stern-Ginossar et al. 2007) and gastric cancer (Shin and Chu 2014), respectively . Furthermore, miRNAs with functional roles in promoting bone metastatic progression could become important therapeutic targets (Waldman and Terzic 2008).

MiR-9 is produced from three independent genes (miR-9-1, miR-9-2, and miR-9-3). Significant upregulation of miR-9 levels were observed in osteosarcoma and hepatocellular carcinoma with poor prognosis (Press 2016). MiR-9 is one of the most abundant miRNAs in the developing and adult brain. Present in the nervous system where they have been shown to influence neuronal development and function (Giusti et al. 2014). It has also been reported to be play an important role in several malignant cancers, such as ovarian, gastric, renal, colon, breast, esophageal, and lung cancer, and brain tumors. Qi et al. (2016) associated miR-9 expression with proliferation, apoptosis, migration and invasion, in OS (osteosarcoma) tissues and cell lines (Qi et al. 2016).

Conclusion

Given the wide spectrum of effects linked to miR-9-5p, we believe it has a relevant role in gene regulations, but additional studies are needed to see how useful it could be as a biomarker or therapeutic target in primary brain calcification.

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References

Aleksic P, Mavroplakis M, Papadopoulos GL et al (2009) Lost in translation: an assessment and perspective for computational microRNA target identification. Bioinformatics 25:3049–3055. doi:10.1093/bioinformatics/btp565

Bottger P, Pedersen L (2002) Two highly conserved glutamate residues critical for type III sodium-dependent phosphate transport revealed by uncoupling transport function from retroviral receptor function. J Biol Chem 277:42741–42747. doi:10.1074/jbc.M207006200

Bottger P, Pedersen L (2011) Mapping of the minimal inorganic phosphate transporting unit of human PiT2 suggests a structure universal to PiT-related proteins from all kingdoms of life. BMC Biochem 12:21. doi:10.1186/1471-2091-12-21

Bottger P, Hoda SE, Grunnet M et al (2006) Characterization of transport mechanisms and determinants critical for Na+ −dependent Pi symport of the PiT family paralogs human PiT1 and PiT2. Am J Physiol Cell Physiol 291:C1377–C1387. doi:10.1152/ajpcell.00152.2006

Burney RO, Hamilton AE, Aghajanova L et al (2009) MicroRNA expression profiling of eutopic secretory endometrium in women with versus without endometriosis. Mol Hum Reprod 15:625–631. doi:10.1093/molehr/gap068

Cao D-D, Li L, Chan W-Y (2016) MicroRNAs: key regulators in the central nervous system and their implication in neurological diseases. Int J Mol Sci 17:842. doi:10.3390/IJMS17060842

Chan X, Zhu I, Ma Z et al (2015) Oncogenic miR-9 is a target of crizotinib in NSCLCs. Sci Rep 5:17031. doi:10.1038/srep17031

Coolen M, Katz S, Bally-Cuif L (2013) miR-9: a versatile regulator of neurogenesis. Front Cell Neurosci 7:220. doi:10.3389/fncel.2013.00220

Giusti SA, Vogl AM, Brockmann MM et al (2014) MicroRNA-9 controls dendritic development by targeting REST. 1–22. doi:10.7554/eLife.02755

Inden M, Iriyama M, Takagi M et al (2013) Localization of type-III sodium-dependent phosphate transporter 2 in the mouse brain. Brain Res 1531:75–83. doi:10.1016/j.brainres.2013.07.058

Jensen N, Schrander HD, Hejbøl EK et al (2013) Loss of function of Slc20a2 associated with familial idiopathic basal ganglia calcification in humans causes brain calcifications in mice. J Mol Neurosci 51:994–9. doi:10.1007/s12031-013-0085-6

Jensen N, Autzen JK, Pedersen L (2016) Slc20a2 is critical for maintaining a physiologic inorganic phosphate level in cerebrospinal fluid. Neurogenetics 17:125–130. doi:10.1007/s10048-015-0469-6

Kavanaugh MP, Miller DG, Zhang W et al (1994) Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. Proc Natl Acad Sci 91:7071–7075. doi:10.1073/pnas.91.15.7071

Keasey MP, Kang SS, Lovins C, Hagg T (2013) Inhibition of a novel spinophilin-integrin signaling pathway increases STAT3-mediated CNTF expression. Cell Commun Signal 11:35. doi:10.1186/1478-811X-11-35

Keasey MP, Lemos RR, Hagg T, Oliveira JRM (2016) Vitamin-D receptor agonist calcitriol reduces calcification in vitro through selective upregulation of SLC20A2 but not SLC20A1 or XPR1. Sci Rep 6:25802. doi:10.1038/srep25802

Keller A, Westenberger A, Sobrido MJ et al (2013) Mutations in the gene encoding PDGFRB cause brain calcifications in humans and mice. Nat Genet 45:1077–82. doi:10.1038/ng.2723

Legati A, Giovannini D, Nicolas G et al (2015) Mutations in XPR1 cause primary familial brain calcification associated with altered phosphate export. Nat Genet 47:579–81. doi:10.1038/ng.3289

Lemos RR, Ramos EM, Legati A et al (2015) Update and mutational analysis of SLC20A2: a major cause of primary familial brain calcification. Hum Mutat 36:489–495. doi:10.1002/humu.22778

Liu W, Zhao N, Li X, et al (2015) A novel FBN2 mutation in a Chinese family with congenital contractural arachnodactyly. 5:163–166

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and. Methods 25:402–408. doi:10.1006/meth.2001.1262

Mccarthy E, Squier W (2014) Patterns and pathways of calcification in the developing brain. 1009–1015. doi:10.1111/dmcn.12493

Nicolas G, Pottier C, Maltête D et al (2013) Mutation of the PDGFRB gene as a cause of idiopathic basal ganglia calcification. Neurology 80:181–187. doi:10.1212/WNL.0b013e31827ccf94

Nitsenko IS, Wellgard R, Nicaudet C et al (2010) Pericytes regulate the blood–brain barrier. 6–11. doi:10.1038/nature09522

Pruss D (2016) Prognostic role of miR-9 expression in various human malignant neoplasms: a meta-analysis. 3039–3047

Qi XJ, Wang JF, Wang GD et al (2016) Pivotal role of microRNA-9 in osteosarcoma tumorigenesis and tumor progression. Genet Mol Res 15:1–10. doi:10.4238/gmr.15017318

Sapio L, Naviglio S (2015) Inorganic phosphate in the development and treatment of cancer: a Janus Bifrons? World J Clin Oncol 6:198–201. doi:10.5306/wjco.v6.i6.198

Shin VY, Chu KM (2014) MiRNA as potential biomarkers and therapeutic targets for gastric cancer. World J Gastrointest Oncol 20:104–52–10439. doi:10.3748/wjg.v20.i20.10432

Stern-Ginossar N, Elefant N, Zimmermann A et al (2007) Host immune system gene targeting by a viral miRNA. Science 317:376–81. doi:10.1126/science.1140956

Ucker W, Willimsky G, Pedersen FS et al (1988) RNA levels of human retrovirus receptor Pit1 and Pit2 do not correlate with infectibility by these retroviral vector pseudotypes. Hum Gene Ther 262:7:2619–2627

Waldman SA, Terzic A (2008) MicroRNA signatures as diagnostic and therapeutic targets. Clin Chem 54:943–944. doi:10.1373/clinchem.2008.105353

Wallingford MC, Chia JJ, Leaf EM et al (2016) SLC20A2 deficiency in mice leads to elevated phosphate levels in cerebrospinal fluid and glymphatic pathway-associated arteriolar calcification, and recapitulates human idiopathic basal ganglia calcification. Brain Pathol 3:64–76. doi:10.1111/bpa.12362

Wang C, Li Y, Shi L et al (2012) Mutations in SLC20A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. Nat Genet 44:254–6. doi:10.1038/ng.1077

Zhang J, Chintalgattu V, Shih T et al (2011) MicroRNA-9 is an activation-induced regulator of PDGFR-beta expression in cardiomyocytes. J Mol Cell Cardiol 51:409–418. doi:10.1016/j.yjmcc.2011.06.001

Zhang H, Shykind B, Sun T (2012) Approaches to manipulating microRNAs in neurogenesis. Front Neurosci 6:1–13. doi:10.3389/fnins.2012.00196

6 ARTIGO 3 : MIR-9-5P REGULATES GENES LINKED TO BRAIN CALCIFICATION IN OSTEOGENIC DIFFERENTIATION MODEL AND LEADS TO A UBIQUITOUS HALT IN ION CHANNELS

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MIR-9-5P REGULATES GENES LINKED TO BRAIN CALCIFICATION IN OSTEOGENIC DIFFERENTIATION MODEL AND LEADS TO A UBIQUITOUS HALT IN ION CHANNELS

Bezerra DP[1], Aguiar JP[2], Keasey M[3], Rodrigues CG[2], Oliveira JRM[1,4*]

1-Keizo Asami Laboratory, Federal University of Pernambuco, Recife, PE, Brazil.
2-Department of Biophysics and Radiobiology, Federal University of Pernambuco, Recife, PE, Brazil.
3-Department of Biomedical Sciences - Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA.
4-Neuropsychiatry Department, Federal University of Pernambuco, Recife, PE, Brazil.

Corresponding author: João Oliveira-Neuropsychiatry Department, Federal University of Pernambuco, Recife, PE, Brazil. joao.ricardo@ufpe.br.

MicroRNA-9 (miR-9) modulates gene expression and present high structural conservation and wide expression in the central nervous system. Bioinformatics analysis predicts almost 100 ion channels, membrane transporters and receptors, including genes linked to primary familial brain calcification (PFBC), as possible miR-9-5p targets. PFBC is a neurodegenerative disorder, characterized by bilateral and symmetrical calcifications in the brain, associated with motor and behavioral disturbances. In this work, we seek to study the influence of miR-9-5p in regulating genes involved in PFBC, in an osteogenic differentiation model with SaOs-2 cells. After characterizing different levels changes in the expression of S*LC20A2*, *PDGFβ* and *PDGFRβ*, we confirmed the findings using a miR-9-5p inhibitor and also probed the cells in an electrophysiological analysis, to access if such microRNA might affect a broader range of ion channels, membrane transporters and receptors. Our electrophysiological data show that the increase of the miR-9-5p in SaOs-2 cells decreased the density and amplitude of the output ionic currents, corroborating that it might influence the activity, and maybe the expression, of some ionic channels. Additional investigations should define if such effect is specific to miR-9-5p and if it could be used, together with the miR-9-5p inhibitor, as therapeutic or diagnostic tool.

Key words: miR-9-5p, brain calcification, SaOs-2 cells, ion channel

Introduction

MicroRNAs (miRNA) regulate many physiological and pathological processes such as cell growth and differentiation, neurogenesis, apoptosis, and tumor invasion, through gene expression modulation. They are non-coding RNAs (21–23 nucleotides long) and act by binding target messenger RNAs (mRNAs), leading to post-transcriptional repression or degradation. MiRNA can have reduced function by miRNAs inhibitors, that bind to mature miRNA, irreversibly, or interpose on biogenesis of miRNA [1].

Amongst many, microRNA-9 (miR-9) is often studied due to its high structural conservation and expression in the central nervous system (CNS)(COOLEN; KATZ; BALLY-CUIF, 2013). MiR-9 can be transcribed from three independent gens MIR9-1(1q22), MIR9-2 (5q14.3) and MIR9-3 (15q26.1), producing two functional forms of miR-9 (miR-9-5p) and miR-9* (miR-9-3p), generated from the 5' strand and 3' strand, respectively(BARBANO et al., 2017). The manipulation of miR-9 expression levels and patterns in the nervous system has demonstrated the critical role they play in neurogenesis (ZHANG; SHYKIND; SUN, 2012) and in different types of cancer (ALEXIOU et al., 2009).

Bioinformatic analysis predicted possible target of miR-9-5p in hundreds of genes, some of them linked to primary familial brain calcification (PFBC) (PAIVA; KEASEY; OLIVEIRA, 2017). Our group is focused in finding cell models to study genes linked to PFBC and an *in vitro* osteogenic differentiation model with SaOs-2 cells coincides with down regulation in PiT1, PiT2 and XPR1(KEASEY et al., 2016). However, we still miss information about other genes linked to PFBC, such as *PDGFRβ* and *PDGFβ*, and how it might be affected by miR-9-5p in a similar osteogenic differentiation model.

PFBC is a rare neurodegenerative disorder, characterized by bilateral calcification in basal ganglia and other brain areas such as cerebellum, thalamic nuclei, and subcortical regions. Symptoms might include parkinsonism, dementia, headaches, mood swings and psychosis. Four genes were linked to familial and sporadic cases, including two inorganic phosphate transporters (*SLC20A2*-enconding PiT2 and *XPR1*) and two genes involved with vascular integrity and pericyte stability (*PDGFRB* and *PDGFB* - platelet-derived growth factor b).

There is a recent interest in developing cell models to study PFBC and to test new therapeutic agents, using different cell lines, including HEK293, SaOs-2 and neuroblastoma SH-SY5Y [5-7].

In this work, we seek to study the influence of miR-9-5p in regulating other genes involved in PFBC, during the calcification process, and also evaluated the cells by electrophysiological techniques to infer how the miR-9-5p can influence the functioning and expression of ion channels.

Results

In silico analyses

Targetscan, a web server (http://www.targetscan.org/vert_71/), was used to search for potential targets for miR-9-5p. Table 1(Supplementary Table S1) lists 27 ion channels, 17 transporters and 40 receptors amongst a total of 1378 targets. We observe two groups of potassium channels, large conductance calcium and voltage-activated potassium, potentially regulated by miR-9-5p and specific inhibitors (data obtained in June 2017)

Osteogenic differentiation in SaOs-2 cells and gene expression analysis

Alizarin Red staining in SaOs-2 cells (Human epithelial-like osteosarcoma cell line) revealed increased mineral deposition in day 7 and day 14 (Fig. 1A), quantify in Figure 2B, under an osteogenic induction protocol (See methods). Gene expression was measured across the differentiation in at least 3 independent experiments. *SLC20A1* and *SLC20A2* showed a significant down-regulation in day 7 (Fig 1C) (31.9% and 32.44%,

respectively) and day 14 (Fig. 1D) (11.7% and 6% respectively). On day 14 (Fig. 1D), *PDGFRβ* and *PDGFB* presented with a 52.8% down-regulation and 63.60% up-regulation.

We thus profiled the expression of miR-9-5p in the same pooled RNA samples as those described above. In the figure 1C, miR-9-5p in three individual groups of cells in triplicates, showed level of expression similar at time 0, however, a tendency to increase was observed on day 14 (Fig.1D) but was not significant. In Fig.1E, the western blot confirmed the decrease to protein expression PiT2 and PDGFrB, on day 14 of differentiation, being more remarkable in PiT2.

MiR-9-5p and miR-9-5p inhibitor in SaOs-2 cells culture regulated SLC20A2 and PDGFRβ

Induced over-expression of miR-9-5p was achieved by transfection of a mimic in SaOs-2 cells, Twenty-four hours' post-transfection, miR-9-5p was increased to ~ $4x10^3$ fold above negative control (Fig.1). Overexpressed of miR-9-5p showed significant decreased levels of *SLC20A2* and *PDGFRβ*, reduced 70% and 44%, respectively (Fig. 2B). In presence of miR-9-5p inhibitor (Fig 2C) could be observed the trend of up-regulating of *SLC20A2*, *PDGFRβ* and *PDGFB*, due to downregulation of miR-9-5p, reduction of 70% expression miR-9-5p (Fig. 2A), approximately, but not significant. *PDGFB* no showed any regulation in presence of miR-9-5p.

Probably, 24h was not enough to result in changes at protein level because PiT2 and PDGFrB proteins had similar expressions that controls (Fig. 2D). On the other hand, both, PiT2 and PDGFrB showed a tendency to increase to expression in presence of miR-9-5p inhibitor (Fig. 2D).

Electrophysiological evidence of miR-9-5p in the regulation expression of potassium channels in SaOs-2 cells

Membrane currents were activated by 500 ms voltage steps from a holding potential of –80 to –100 mV up to +60 mV (10 mV increments, sweep interval 2 s) shown in Figure 3B. Two types of ionic currents activated by voltage steps in SaOs-2 cells are displayed in Figure 3A. In the potential of +60 mV was observed a fast activation component with noisy oscillations similar to a calcium-activated potassium channels (IKCa).

Another component showed a gradually activating current at +20mV and +40mV, like to delayed rectifier K^+ current (IK_{DR}). The exposure of the cells to miR-9-5p reduced approximately 85 % all current profiles (Fig. 3B). In figure 3C even the cells exposed to miR-9-5p inhibitor are observed current profiles with intensity of only 20% in relation to the control. The current density decreased under both conditions in the presence of miR-9-5p or inhibitor miR-9-5p (Fig.3D).

Discussion

In vitro osteogenic differentiation is the process chemically induced by dexamethasone (Dex), ascorbic acid (Asc) and β-glycerophosphate (β-Gly), leading to bone cell like features. Differentiated osteoblasts have extracellular calcium deposits that can be confirmed with alizarin red S stain, alkaline phosphatase activity and specific bone markers such as osteocalcin (OC), transforming growth factor-beta (TGF-b), estrogen receptor-alpha (ER-a), osteopontin (OPN), type I collagen (Col1), matrix bone sialoprotein (MBS) and bone morphogenetic protein (BMPs)(SILA-ASNA et al., 2007) (HUANG et al., 2007) (BIRMINGHAM et al., 2012) (ARPORNMAEKLONG et al., 2009). This methodology is considered a good model to understand the mechanisms of calcification. The mineral deposits in PFBC are visualized as bilateral calcifications characterized, by the accumulation of calcium (Ca^{++}) in hydroxyapatite deposits (calcium phosphate crystals)(MOURA; OLIVEIRA, 2015), around the walls of capillaries, arteries and veins(TAGLIA et al., 2015), however is not clear how different mutations can show the same brain phenotype. The present study performed an osteogenic differentiation model for 14 days that was used to analyze the effects of the mineral deposits on the genes linked to PFBC. The experiments performed in the present work revealed similar results as previously published by Keasey et al 2015, in which the decrease of *SLC20A2* expression was observed after 7 days of differentiation and a lower level was detected after 14 days. On the other hand, the changes found in *SLC20A1* expression can be due to a reduction of β-Gly concentration (10nM to 1nM) in the differentiation protocol. On day 14 of differentiation, *PDGFRβ* reduced expression while, contrary, *PDGFβ* increased expression level suggesting a compensatory relation.

In silico studies can be the first assay to identify miRNA their targets, since prediction directs to better triage and understand a biological function of miRNAs. Usually, the most used programs are PicTar, TargetScan, DIANA microT, MicroCosm and miRanda. The principle of Target Scan is the perfect seed recognition, considering conservation in many genomes and features surrounding mRNA (BERNARDO et al., 2012). In preview studies, *in silico*, we showed that miR-9-5p in predict regulate *SLC20A2* and *PDGFRβ*. This relation was confirmed overexpressing miR-9-5p in HEK293 cells, getting a downregulation of PiT2 indicating an inverse relation(PAIVA; KEASEY; OLIVEIRA, 2017). That relation can be observed in calcification process of SaOs-2 cells, in addition to decreased of SLC20A2 expression, *PDGFRβ* have the same behavior through increasing the expression of miR-9-5p. Our findings, in SaOs-2 cells, ratify that interaction where overexpression of miR-9-5p change the profile of expression of PiT2 and PDGFrB, and down-expression of miR-9-5p attenuated the inhibitory effect, in PiT2 and PDGFrB, by reverse the action of miR-9-5p. Two factors could explain the inhibitory effect is not so effective: time and concentration. The exposure for a short period of time (24h) of SaOs-2 cells to miR-9-5p inhibitor and the concentration (30nM) may not be enough to revert the regulatory role of miRNA endogenous. Mutation in *SLC20A2* and *PDGFRβ* are mains responsible for the number of patients with PFBC, SLC20A2 (PiT2) accounts for about 54% of cases, 40 % of familial form and 14 % of sporadic cases, while P *PDGFRβ* and *PDGFβ* mutations are likely rare (TAGLIA et al., 2015). Differentiation osteogenic in SaOs-2 cells is an attractive model do study PFBC, because mimetic the molecular status of patients, reducing the expression of the main genes involved.

MiRNAs regulate numerous biological processes through of translation and degradation of target gene expression by binding at 3′UTR and coding regions of their target mRNAs by an imperfect base pairing (Watson-Crick), involved in developmental and physiological processes, and altered expression is linked to human diseases (FELEKKIS et al., 2010). Among the miRNA, miR-9-5p has a sequence conserved, expressed differently between species, highly and specifically expressed in NPCs (neural progenitor cells) (YUVA-AYDEMIR et al., 2011) and CNS (central nervous system), regulating proliferation and differentiation process(COOLEN; KATZ; BALLY-CUIF, 2013).

In this work, multiple possible targets, including transports, receptors, and ion channels, are predicted to be regulated by miR-9-5p. Others studies identified miR-9-5p regulating different ion channels. KCNMA1 gene transcribes pore-forming α subunit of the BK channel, known as MaxiK, a large-conductance calcium- and voltage-activated potassium channel (PIETRZYKOWSKI et al., 2008), and sodium voltage-gated channels (SCN) Gene, β2-subunit of voltage-gated sodium channels (Navβ2)(SUN et al., 2015) levels of both channels in brain regions, in adverse conditions, were negatively regulated while miR-9-5p expression increased. Data involving functional point of view between miR-9-5p and ion channels are scarce in literature.

BK channels play a central role in the regulation of neuronal excitability, controlling neurotransmitter release and the shaping of action potentials in many brain regions. On a cellular level, BK serves as an integrator of regulatory processes, because it is activated by both voltage and intracellular calcium(SHIEH et al., 2000). This channel has a very high conductance, and sustained activation would likely have serious consequences for nervous system function(GE et al., 2014).

Our electrophysiological data show that a miR-9-5p increase (Fig 3B and Fig 3D) in SaOs-2 cells decreases the current density (amplitude) of the output currents, which include the BK channels. One of the mechanisms that could explain this behavior was suggested in the modeling study carried out by Pietrzykowski et al, 2008 when they associated the alcohol tolerance involving miRNA. Clinically relevant alcohol concentrations rapidly increased miR-9-5p levels in central neurons within minutes of alcohol exposure. Only one out of three BK channel 3' UTRs contains an miR-9-5p Recognition Element (MRE) with complementarity to miR-9-5p. Thus, there is a selective degradation of mRNA resulting in reorganization of BK splice variant profile. Therefore, the increase in miR-9-5p causes a reduction in the number of BK channels. The miRNA interaction with its target mRNA usually results in downregulation of that target, or translation repression (PIETRZYKOWSKI et al., 2008).
One siRNA molecule with miRNA-like features can degrade several target molecules in a very short time [19,](ZAMORE, [s.d.])(KYLE et al., 2013) . This could also explain why we observe such a massive downregulation of BK mRNA in a very short period of time (Fig.3D).

In addition, suppression of the actions of miR-9-5p with specific inhibitor could help to establish the consequences of its regulation (Fig. 3C).

There are already some reports in which miR-9-5p controls the progression of neurogenesis (KRICHEVSKY et al., 2006), (LEUCHT et al., 2008), (DELALOY et al., 2010), (BONEV; PISCO; PAPALOPULU, 2011), (COOLEN et al., 2012; COOLEN; KATZ; BALLY-CUIF, 2013) mainly in the development of zebrafish (COOLEN et al., 2012; LEUCHT et al., 2008) and in models with Human Embryonic Stem Cell-Derived Neural (DELALOY et al., 2010). In general, in these studies, suppression of miR-9-5p activity promoted a delay in cell proliferation.

It is already known that ion channels can also contribute significantly to cell mitotic biochemical signaling, cell cycle progression, as well as cell volume regulation. These functions are critically important for the proliferation of cells, including cancer cells (LANG et al., 2005; RAO et al., 2015). Potassium channels play a key role in this process (GLASSMEIER et al., 2012; JANG et al., 2009; KACZMAREK, 2006; URREGO et al., 2014; WANG et al., 2007; YASUDA; CUNY; ADAMS, 2013).

In this context, the absence of miR-9 is likely to retard cell proliferation, via ion channels, by paralyzing / blocking the cells in the G0 / G1 phase of the cell cycle, where the permeability to K^+ ions is lower (RAO et al., 2015). This could explain one of the mechanisms by which the potassium current density in SaOs-2 cells is decreased in the presence of the miR-9-5p inhibitor compared to the control (Fig.3D). Recently in another study it has been shown that miR-26a also delays cell proliferation, but by another Wnt / β catenin signaling pathway (QU et al., 2015).

Methods

Bioinformatics prediction

The binding affinity of the miR-9-5p to genes link to PFBC and ion channel, transporters and receptors was predicted using the online algorithms TargetScan is available online at http://www.targetscan.org/docs/UTR_profiles.html.

Cell culture

SaOs-2 cells were obtained from the Rio de Janeiro Cell Bank. The cells were cultured in Dulbecco's modified eagle medium (DMEM) (Cat # 12100046, Gibco) supplemented with 10% fetal bovine serum (SFB) (Cat # 10437028, Gibco), 1mM glutamine (Cat #25030081, Gibco) and 10,000 U/mL penicillin and streptomycin (Cat# 15140122 Invitrogen, USA). Cells passaged every 3 days and maintained at 37 °C with 5% CO2 with approximately 95% humidity.

Transfections

Cells were seeded onto 6-well plates ($1x10^5$ cells per mL). After 24hr (50% confluent), the medium was removed and stored for later use (Conditioned media). SaOs-2 cells were transfected with miR-9-5p (30nM, Cat# PM10022, Ambion) or miR-9-5p inhibitor (30nM, Cat#MIMAT0000142, Dharmacon). All transfections were carried out in triplicate with Lipofectamine 2000 (Cat# 11668027, Invitrogen) and Opti-MEM medium (Cat#31985070, Gibco). After 4 Hours, medium was removed and replaced with the conditioned media. RNA and Protein were extracted after 24 hours.

Osteogenic induction

Approximately, $2x10^5$ cells were placed 6-well plates and $2.5x10^4$ in 96-well. The osteogenic medium composing of the maintenance medium added 250 μM ascorbic acid (Sigma), 1 mM β-glycerophosphate (Sigma). The medium of the cultured cells was replaced every 3 days, except ascorbic acid that was added every day. Identification and

quantification of osteoblast markers (cytochemistry staining) were done on days 0, 7 and 14. Cytochemistry staining of osteogenic induced was done with Alizarin red in 96 wells. Initially, the medium was removed, and the cell layers were fixed with PA 4% for 15 minutes. After, the cells were washed with destiled water e added the alizarin for 20 minutes. The stain was removed and the cell washed multiple times. Some images were made before to make the quantification of staining. The quantification was done with cetylpyridinum chloride 5%. After well-washed add cetylpyridinum chloride 5% 100µL/well, for 10 minutes, in the stained plate. Remove 100µL in plate 96 well and read in spectrometer at 570nm.

Western blot

Protein isolation was performed according to Keasey et al (KEASEY et al., 2013). Antibodies used were PiT2 (sc-377326 - 1:1000, Santa Cruz, CA, USA) and GAPDH (sc-25778, 1:1000, Santa Cruz). Signal was developed with a HRP conjugated anti-rabbit (sc-2030) and anti-mouse (sc-2005) secondary antibody and ECL substrate (Cat# WP20005, Invitrogen) in Chemidoc (Bio-Rad). PiT2 and PDGFrB protein levels were quantified by densitometry using the ImageJ system, and expressed as ratio relative to GAPDH.

Real time qPCR (qRT-PCR)

RNA isolation, cDNA and qPCR were performed according to Keasey et al (KEASEY et al., 2016). With 0.5µL of 20× TaqMan Gene Expression Assay (GAPDH, #Hs02758991_g1; SLC20A2, #Hs00198840_m1; SLC20A1, #Hs00965587_m1; FBN2 #Hs00266592_m1; PDGFrB #Hs00387364_m1; miR-9-5p #PN4427975; U6

#PN4427975_m1 from Applied Biosystems). The normalized relative transcription levels were calculated by $2^{-\Delta\Delta CT}$method(LIVAK; SCHMITTGEN, 2001).

Electrophysiology

Electrophysiological recording was performed in the whole-cell configuration using a patch clamp amplifier (Axopatch 200B; Axonm Instruments/Molecular Devices Corp., Union City, CA, http://www. moleculardevices.com). Borosilicate glass electrodes (1.2-mm outside diameter; Warner Instruments, Hamden, CT, http://www. warneronline.com) were pulled with a vertical pipette puller (model PP-830; Narishige, Tokyo, http://www.narishige.co.jp) and had tip resistances of 2–3 MΩ when filled with pipette solution. After a giga-seal was obtained by negative suction, the cell membrane was ruptured by gentle suction to establish the whole-cell configuration. All recordings were performed at room temperature. The sampling rate used was 500,000 kHz and the recorded signal was filtered at 2 kHz and transferred to a computer using the Digidata 1550A interface (Axon Instruments). Acquired whole cell current data were analyzed with the pCLAMP program (version 10.5; Axon Instruments). For electrophysiological recordings, the cells were transferred to a small chamber and allowed to attach to the glass bottom for 30 minutes. The suspension was stored at room temperature and used within 4-6 hours. Electrophysiological recordings were performed using ball-shaped cells obtained after trypsin-EDTA treatment of the cultures. We used 10mM Tetraethylammonium (TEA) (Sigma, St Louis, MO, USA) an inhibitor of voltage-gated K+ channels.

Experimental Solutions

The normal Tyrode solution contained 140 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl2, 2,0 mM CaCl2, 10 mM glucose, and 10 mM HEPES; the pH was adjusted to 7.4 with NaOH. The pipette solution contained 130 mM KCl, 10mM NaCl, 10 mM HEPES, 5.0 mM EGTA; the pH was adjusted to 7.2 with KOH. Both intracellular and extracellular solutions are filtered by a 0.22 μm filter.

Statistics

Data were analysed using GraphPad Prism (Version 5a) software using a One-Way Anova with a Dunnett's post-hoc test ($p < *0.05$ and **0.01).

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References

ALEXIOU, P. et al. Lost in translation: An assessment and perspective for computational microrna target identification. **Bioinformatics**, v. 25, n. 23, p. 3049–3055, 2009.

ARMULIK, A. et al. Pericytes regulate the blood–brain barrier. **Nature**, v. 468, n. 7323, p. 557–561, 25 nov. 2010.

ARPORNMAEKLONG, P. et al. Phenotypic Characterization, Osteoblastic Differentiation, and Bone Regeneration Capacity of Human Embryonic Stem Cell–Derived Mesenchymal Stem Cells. **Stem Cells and Development**, v. 18, n. 7, p. 955–968, 2009.

ARTS, F. A. et al. Idiopathic basal ganglia calcification-associated\n PDGFRB\n mutations impair the receptor signalling. **Journal of Cellular and Molecular Medicine**, v. 19, n. 1, p. 239–248, 2015.

BARBANO, R. et al. Stepwise analysis of MIR9 loci identifies miR-9-5p to be involved in Oestrogen regulated pathways in breast cancer patients. **Scientific Reports**, v. 7, n. March, p. 45283, 2017.

BATLA, A. et al. Deconstructing Fahr's disease/syndrome of brain calcification in the era of new genes. **Parkinsonism & Related Disorders**, v. 37, p. 1–10, abr. 2017.

BERNARDO, B. C. et al. A MicroRNA Guide for Clinicians and Basic Scientists: Background and Experimental Techniques. **Heart Lung and Circulation**, v. 21, n. 3, p. 131–142, 2012.

BIRMINGHAM, E. et al. Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. **European Cells and Materials**, v. 23, n. 353, p. 13–27, 2012.

BLAINE, J.; CHONCHOL, M.; LEVI, M. Renal control of calcium, phosphate, and magnesium homeostasis. **Clinical Journal of the American Society of Nephrology**, v. 10, n. 7, p. 1257–1272, 2015.

BONEV, B.; PISCO, A.; PAPALOPULU, N. MicroRNA-9 Reveals Regional Diversity of Neural Progenitors along the Anterior-Posterior Axis. **Developmental Cell**, v. 20, n. 1, p. 19–32, 2011.

BØTTGER, P. et al. Characterization of transport mechanisms and determinants critical for Na+-dependent Pi symport of the PiT family paralogs human PiT1 and PiT2. **American journal of physiology. Cell physiology**, v. 291, n. 6, p. C1377–C1387, 2006.

CHEN, X. et al. Oncogenic miR-9 is a target of erlotinib in NSCLCs. **Sci Rep**, v. 5, p. 17031, 2015.

COOLEN, M. et al. MiR-9 Controls the Timing of Neurogenesis through the Direct Inhibition of Antagonistic Factors. **Developmental Cell**, v. 22, n. 5, p. 1052–1064, 2012.

COOLEN, M.; KATZ, S.; BALLY-CUIF, L. miR-9: a versatile regulator of neurogenesis. **Frontiers in Cellular Neuroscience**, v. 7, n. November, p. 220, 2013.

DANEMAN, R. et al. Pericytes are required for blood–brain barrier integrity during embryogenesis. **Nature**, v. 468, n. 7323, p. 562–566, 2010.

DANG, C. V. et al. Function of the c-Myc Oncogenic Transcription Factor. **Experimental Cell Research**, v. 253, n. 1, p. 63–77, 1999.

DE OLIVEIRA, J. R. M.; DE OLIVEIRA, M. F. Basal ganglia calcification as a putative cause for cognitive decline. **Dementia & Neuropsychologia**, v. 7, n. 2, p. 151–154, 2013.

DELALOY, C. et al. MicroRNA-9 Coordinates Proliferation and Migration of Human Embryonic Stem Cell-Derived Neural Progenitors. **Cell Stem Cell**, v. 6, n. 4, p. 323–335, abr. 2010.

DRAKAKI, A. et al. Functional microRNA high throughput screening reveals miR-9 as a central regulator of liver oncogenesis by affecting the PPARA-CDH1 pathway. **BMC Cancer**, v. 15, n. 1, p. 542, 2015.

DUMONT, J. et al. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. **Critical Reviews in Biotechnology**, v. 36, n. 6, p.

1110–1122, 2016.

EBERT, M. S.; NEILSON, J. R.; SHARP, P. A. NIH Public Access. v. 4, n. 9, 2013.

FELEKKIS, K. et al. MicroRNAs: A newly described class of encoded molecules that play a role in health and disease. **Hippokratia**, v. 14, n. 4, p. 236–240, 2010.

FERREIRA, D.; ADEGA, F.; CHAVES, R. The Importance of Cancer Cell Lines as in vitro Models in Cancer Methylome Analysis and Anticancer Drugs Testing. **Oncogenomics and Cancer Proteomics- Novel Approaches in Biomarkers Discovery and Therapeutic Targets in Cancer**, p. 139–166, 2013.

GAGLIARDI, M. et al. A SLC20A2 mutation identified in an asymptomatic patient with brain calcification. **Journal of the Neurological Sciences**, v. 372, p. 70–72, 2017.

GE, L. et al. Big Potassium (BK) ion channels in biology, disease and possible targets for cancer immunotherapy. **International Immunopharmacology**, v. 22, n. 2, p. 427–443, out. 2014.

GLASSMEIER, G. et al. Inhibition of HERG1 K + channel protein expression decreases cell proliferation of human small cell lung cancer cells. **Pflugers Archiv European Journal of Physiology**, v. 463, n. 2, p. 365–376, 2012.

HUANG, W. et al. Signaling and transcriptional regulation in osteoblast commitment and differentiation. **Frontiers in bioscience**, v. 12, p. 3068–92, 2007.

INDEN, M. et al. Localization of type-III sodium-dependent phosphate transporter 2 in the mouse brain. **Brain Research**, v. 1531, p. 75–83, 2013.

JANG, S. H. et al. Silencing of Kv4.1 potassium channels inhibits cell proliferation of tumorigenic human mammary epithelial cells. **Biochemical and biophysical research communications**, v. 384, n. 2, p. 180–6, 2009.

JENSEN, N. et al. Loss of function of Slc20a2 associated with familial idiopathic Basal Ganglia calcification in humans causes brain calcifications in mice. **Journal of molecular neuroscience**, v. 51, n. 3, p. 994–9, 2013.

KACZMAREK, L. K. Non-conducting functions of voltage-gated ion channels. **Nature Reviews Neuroscience**, v. 7, n. 10, p. 761–771, 2006.

KAUR, P.; ARMUGAM, A.; JEYASEELAN, K. MicroRNAs in Neurotoxicity. **Journal of toxicology**, v. 2012, p. 870150, 2012.

KEASEY, M. P. et al. Inhibition of a novel specific neuroglial integrin signaling pathway increases STAT3-mediated CNTF expression. **Cell communication and signaling : CCS**, v. 11, n. 1, p. 35, 2013.

KEASEY, M. P. et al. Vitamin-D receptor agonist calcitriol reduces calcification in vitro through selective upregulation of SLC20A2 but not SLC20A1 or XPR1. **Scientific Reports**, v. 6, n. April, p. 25802, 2016.

KELLER, A. et al. Mutations in the gene encoding PDGF-B cause brain calcifications in humans and mice. **Nature genetics**, v. 45, n. 9, p. 1077–82, 2013.

KIM, V. N. MicroRNA biogenesis: coordinated cropping and dicing. **Nature reviews.**

Molecular cell biology, v. 6, n. 5, p. 376–85, 2005.

KOZAK, C. A. The mouse "xenotropic" gammaretroviruses and their XPR1 receptor. **Retrovirology**, v. 7, n. 1, p. 101, 2010.

KRICHEVSKY, A. M. et al. Specific MicroRNAs Modulate Embryonic Stem Cell-Derived Neurogenesis. **STEM CELLS**, v. 24, n. 4, p. 857–864, abr. 2006.

KYLE, B. D. et al. regulated by Cysteine String Protein. p. 29–32, 2013.

LANG, F. et al. Ion channels in cell proliferation and apoptotic cell death. **Journal of Membrane Biology**, v. 205, n. 3, p. 147–157, 2005.

LEGATI, A. et al. Mutations in XPR1 cause primary familial brain calcification associated with altered phosphate export. **Nature genetics**, v. 47, n. 6, p. 579–81, 2015a.

LEGATI, A. et al. Mutations in XPR1 cause primary familial brain calcification associated with altered phosphate export. **Nature genetics**, v. 47, n. 6, p. 579–81, 2015b.

LEMOS, R. R. et al. Update and mutational analysis of SLC20A2: A major cause of primary familial brain calcification. **Human Mutation**, v. 36, n. 5, p. 489–495, 2015.

LEUCHT, C. et al. MicroRNA-9 directs late organizer activity of the midbrain-hindbrain boundary. **Nature Neuroscience**, v. 11, n. 6, p. 641–648, 2008.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and. **Methods**, v. 25, p. 402–408, 2001.

LUKIW, W. J. et al. Common micro RNAs (miRNAs) target complement factor H (CFH) regulation in Alzheimer's disease (AD) and in agerelated macular degeneration (AMD). **International Journal of Biochemistry and Molecular Biology**, v. 3, n. 1, p. 105–116, 2012.

M. WITKOS, T.; KOSCIANSKA, E.; J. KRZYZOSIAK, W. Practical Aspects of microRNA Target Prediction. **Current Molecular Medicine**, v. 11, n. 2, p. 93–109, 2011.

MACK, B. et al. Rapid and Non-Enzymatic In Vitro Retrieval of Tumour Cells from Surgical Specimens. **PLoS ONE**, v. 8, n. 1, p. 1–9, 2013.

MANYAM, B. V. What is and what is not "Fahr"s disease'. **Parkinsonism & Related Disorders**, v. 11, n. 2, p. 73–80, mar. 2005.

MANYAM, B. V.; WALTERS, A. S.; NARLA, K. R. Bilateral Striopallidodentate calcinosis: Clinical characteristics of patients seen in a registry. **Movement Disorders**, v. 16, n. 2, p. 258–264, 2001.

MORI, K. et al. Dexamethasone Enhances In Vitro Vascular Calcification by Promoting Osteoblastic Differentiation of Vascular Smooth Muscle Cells. p. 1–4, 1999.

MOURA, D. A. P.; OLIVEIRA, J. R. M. XPR1: a Gene Linked to Primary Familial Brain Calcification Might Help Explain a Spectrum of Neuropsychiatric Disorders. **Journal of Molecular Neuroscience**, v. 57, n. 4, p. 519–521, 1 dez. 2015.

MUFADDEL, A. A.; AL-HASSANI, G. A. Familial idiopathic basal ganglia calcification (Fahr`s disease). **Neurosciences (Riyadh, Saudi Arabia)**, v. 19, n. 3, p. 171–7, 2014.

NICOLAS, G. et al. Mutation of the PDGFRB gene as a cause of idiopathic basal ganglia calcification. **Neurology**, v. 80, n. 2, p. 181–187, 2013.

NOWEK, K. et al. Expression of a passenger miR-9* predicts favorable outcome in adults with acute myeloid leukemia less than 60 years of age. **Leukemia**, v. 30, n. 2, p. 303–309, 2016.

OLIVEIRA, J. R. M.; OLIVEIRA, M. F. **Primary brain calcification in patients undergoing treatment with the biphosphanate alendronate.Scientific reports**, 2016. Disponível em: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4792151&tool=pmcentrez&rendertype=abstract>

PAIVA, D. P.; KEASEY, M.; OLIVEIRA, J. R. M. MiR-9-5p Down-Regulates PiT2, but not PiT1 in Human Embryonic Kidney 293 Cells. **Journal of Molecular Neuroscience**, v. 62, n. 1, p. 28–33, 2017.

PAUTKE, C. et al. Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. **Anticancer Research**, v. 24, n. 6, p. 3743–3748, 2004.

PIETRZYKOWSKI, A. Z. et al. Posttranscriptional Regulation of BK Channel Splice Variant Stability by miR-9 Underlies Neuroadaptation to Alcohol. **Neuron**, v. 59, n. 2, p. 274–287, 2008.

PIMENTEL, L. F.; LEMOS, R. R.; OLIVEIRA, J. R. Phosphate Transporters Expression in Patients with Primary Familial Brain Calcifications. **Journal of Molecular Neuroscience**, 2017.

PRESS, D. Fahr ’ s disease : current perspectives. p. 43–49, 2015.

QU, F. et al. MicroRNA‐26a induces osteosarcoma cell growth and metastasis via the Wnt/β‐catenin pathway. **Oncology Letters**, p. 1592–1596, 2015.

RAICA, M.; CIMPEAN, A. M. Platelet-derived growth factor (PDGF)/PDGF receptors (PDGFR) axis as target for antitumor and antiangiogenic therapy. **Pharmaceuticals**, v. 3, n. 3, p. 572–599, 2010.

RAO, V. R. et al. Voltage-gated ion channels in cancer cell proliferation. **Cancers**, v. 7, n. 2, p. 849–875, 2015.

RODAN, S. B. et al. Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties. **Cancer Research**, v. 47, n. 18, p. 4961–4966, 1987.

ROESE-KOERNER, B. et al. Reciprocal Regulation between Bifunctional miR-9/9??? and its Transcriptional Modulator Notch in Human Neural Stem Cell Self-Renewal and Differentiation. **Stem Cell Reports**, v. 7, n. 2, p. 207–219, 2016.

RUPAIMOOLE, R.; SLACK, F. J. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. **Nature Reviews Drug Discovery**, 2017.

SALEEM, S. et al. Fahr's syndrome: literature review of current evidence. **Orphanet journal of rare diseases**, v. 8, n. 1, p. 156, 2013.

SAPIO, L.; NAVIGLIO, S. Inorganic phosphate in the development and treatment of cancer: A Janus Bifrons? **World journal of clinical oncology**, v. 6, n. 6, p. 198–201, 2015.

SHARMA, P. et al. RANKL-RANK signaling regulates expression of xenotropic and polytropic virus receptor (XPR1) in osteoclasts. **Biochemical and Biophysical Research Communications**, v. 399, n. 2, p. 129–132, 2010.

SHIEH, C.-C. et al. Potassium Channels: Molecular Defects, Diseases, and Therapeutic Opportunities. **Pharmacol. Rev.**, v. 52, n. 4, p. 557–594, 2000.

SILA-ASNA, M. et al. Osteoblast differentiation and bone formation gene expression in strontium-inducing bone marrow mesenchymal stem cell. **The Kobe journal of medical sciences**, v. 53, n. 1–2, p. 25–35, 2007.

SIOMI, H.; SIOMI, M. C. Posttranscriptional Regulation of MicroRNA Biogenesis in Animals. **Molecular Cell**, v. 38, n. 3, p. 323–332, 14 maio 2010.

SOBRIDO, M. J. et al. Primary Familial Brain Calcification. **GeneReviews(®)**, p. 1–19, 2014.

SORIANO, P. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. **Genes & Development**, v. 8, n. 16, p. 1888–1896, 1994.

STAPPERT, L.; ROESE-KOERNER, B.; BRÜSTLE, O. The role of microRNAs in human neural stem cells, neuronal differentiation and subtype specification. **Cell and Tissue Research**, v. 359, n. 1, p. 47–64, 2015.

STENVANG, J. et al. Inhibition of microRNA function by antimiR oligonucleotides. **Silence**, v. 3, n. 1, p. 1–17, 2012.

SUN, L.-H. et al. MicroRNA-9 induces defective trafficking of Nav1.1 and Nav1.2 by targeting Navβ2 protein coding region in rat with chronic brain hypoperfusion. **Molecular Neurodegeneration**, v. 10, n. 1, p. 36, 2015.

TADIC, V. et al. Primary Familial Brain Calcification With Known Gene Mutations. **JAMA Neurology**, v. 72, n. 4, p. 460, 2015.

TAGLIA, I. et al. Primary familial brain calcification: update on molecular genetics. **Neurological Sciences**, v. 36, n. 5, p. 787–794, 2015.

TO, C. Gastrointestinal Absorption of Heparins. n. November, p. 1–22, 2009.

TOPOL, A. et al. Dysregulation of miRNA-9 in a Subset of Schizophrenia Patient-Derived Neural Progenitor Cells. **Cell Reports**, v. 15, n. 5, p. 1024–1036, 2016.

URREGO, D. et al. Potassium channels in cell cycle and cell proliferation. **Philosophical transactions of the Royal Society of London. Series B, Biological sciences**, v. 369, n. 1638, p. 20130094, 2014.

WAHID, F. et al. MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. **Biochimica et Biophysica Acta - Molecular Cell Research**, v. 1803, n. 11, p.

1231–1243, 2010.

WALLINGFORD, M. C. et al. SLC20A2 Deficiency in Mice Leads to Elevated Phosphate Levels in Cerbrospinal Fluid and Glymphatic Pathway-Associated Arteriolar Calcification, and Recapitulates Human Idiopathic Basal Ganglia Calcification. **Brain Pathology**, v. 3, n. 28, p. 64–76, 2016.

WALLINGFORD, M. C.; GAMMILL, H. S.; GIACHELLI, C. M. Slc20a2 deficiency results in fetal growth restriction and placental calcification associated with thickened basement membranes and novel CD13 and lamininα1 expressing cells. **Reproductive Biology**, v. 16, n. 1, p. 13–26, 2016.

WANG, C. et al. Mutations in SLC20A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. **Nature genetics**, v. 44, n. 3, p. 254–6, 2012.

WANG, Y.; BLELLOCH, R. NIH Public Access. **Cancer**, v. 69, n. 10, p. 4093–4096, 2010.

WANG, Z. H. et al. Blockage of intermediate-conductance-Ca2+-activated K+ channels inhibits progression of human endometrial cancer. **Oncogene**, v. 26, n. 35, p. 5107–5114, 2007.

WIDER, C. et al. Familial idiopathic basal ganglia calcification: A challenging clinical-pathological correlation. **Journal of Neurology**, v. 256, n. 5, p. 839–842, 2009.

WINTER, J. et al. Many roads to maturity: microRNA biogenesis pathways and their regulation. **Nature cell biology**, v. 11, n. 3, p. 228–234, 2009.

YANG, L. et al. Involvement of miR-9/MCPIP1 axis in PDGF-BB-mediated neurogenesis in neuronal progenitor cells. **Cell death & disease**, v. 4, n. 12, p. e960, 2013.

YASUDA, T.; CUNY, H.; ADAMS, D. J. K_v 3.1 channels stimulate adult neural precursor cell proliferation and neuronal differentiation. **The Journal of Physiology**, v. 591, n. 10, p. 2579–2591, 2013.

YUVA-AYDEMIR, Y. et al. MicroRNA-9. **RNA Biology**, v. 8, n. 4, p. 557–564, 27 jul. 2011.

ZAMORE, P. D. A Micr oRNA in a Multiple-Tur nover RNAi Enzyme Complex. n. 3, p. 1–8, [s.d.].

ZAWISTOWSKI, J. S. et al. MicroRNA 9-3p Targets 1 Integrin To Sensitize Claudin-Low Breast Cancer Cells to MEK Inhibition. **Molecular and Cellular Biology**, v. 33, n. 11, p. 2260–2274, 2013.

ZHANG, H.; SHYKIND, B.; SUN, T. Approaches to manipulating micrornas in neurogenesis. **Frontiers in Neuroscience**, v. 6, n. January, p. 1–13, 2012.

ZHANG, J. et al. MicroRNA-9 is an activation-induced regulator of PDGFR-beta expression in cardiomyocytes. **Journal of Molecular and Cellular Cardiology**, v. 51, n. 3, p. 409–418, 2011.

ZIMMERMAN, A. L.; WU, S. MicroRNAs, cancer and cancer stem cells. **Cancer**

Letters, v. 300, n. 1, p. 10–19, jan. 2011.

Figures

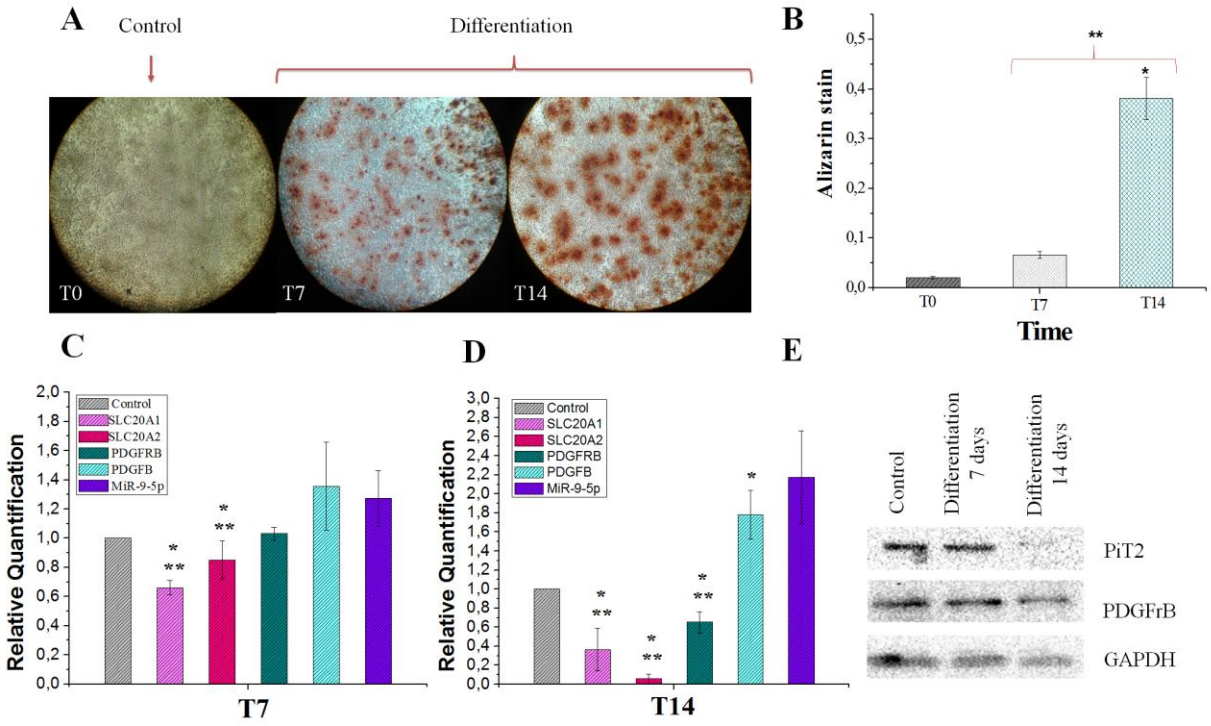

Figure1. Osteogenic differentiation in SaOs-2 cells express different profile of genes. SaOs-2 cells were osteogenic differentiation (**A**), in T0, the control to experiment, wasn't stain to Alizarin Red staining. On the other hand, T7 and T14 were positive Alizarin Red, after chemistry induction, and quantified with Cetylpyridinium chloride (**B**). qRT-PCR analysis for mRNA expression during the course of differentiation in 4 independent experiments, except miR-9-5p (3 independent experiments). Relative expression of SLC20A1, SLC20A2, PDGFRB, PDGFB and MiR-9-5p was measured by qRT-PCR in T7 (**C**) and T14 (**D**). SLC20A1 and SLC20A2 expression in cell differentiation in T7 e T14 was decreased. In T14, PDGFRB and PDGFB showed an antagonistic profile, where PDGFB increased an expression and PDGFRB decreases, significantly. MiR-9-5p showed a tendency to increased. Western blot analysis (**E**) of PiT2 and PDGFrB proves the protein expression in T7 and T14 differentiation days. The bar graphs show mean ± SEM, *P < 0.05 and **P < 0.01 compared to control cells versus non-induced group.

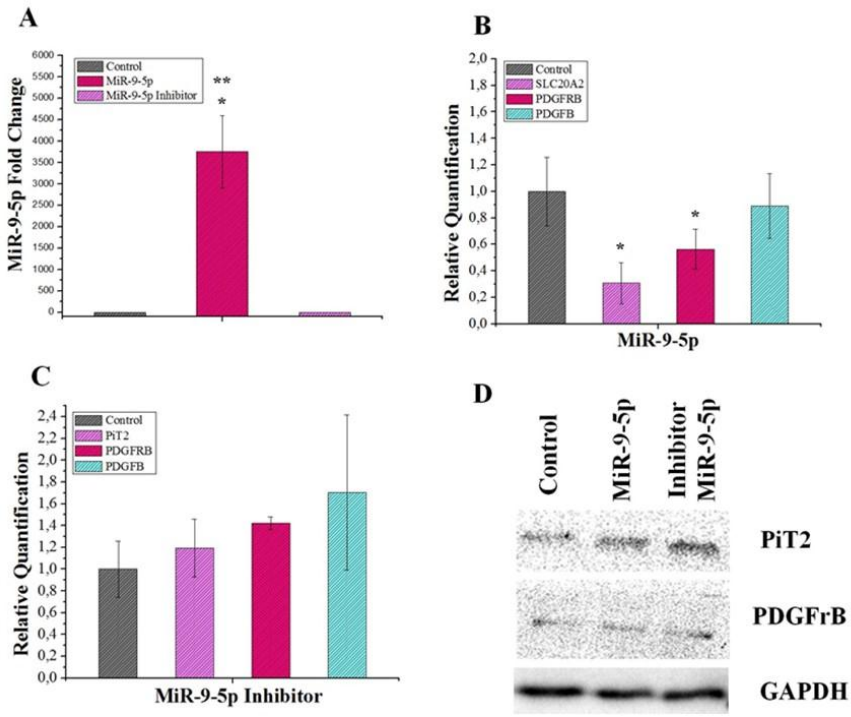

Figure 2. Overexpression of *miR-9-5p* reduces *SLC20A2* and *PDGFRB* expression in SaOs-2. In A, miR-9-5p (30nM) and miR-9-5p inhibitor (30nM) transfected in SaOs-2 cells expression was assayed by qRT-PCR, after 24h. The overexpression of miR-9-5p showed altered mRNA expression in *SLC20A2* and *PDGFRB* (**B**), reduced both expression, but no significant change was seen in cells treated with miR-9-5p inhibitor (**C**). (**D**) Western blot analysis of PiT2 and PDGFrB expression in cells transfected with 30-nM of miR-9-5p and miR-9-5p inhibitor. The *bar graphs* show mean ± SEM, $*P < 0.05$ and $**P < 0.01$ compared to control cells versus non-induced group.

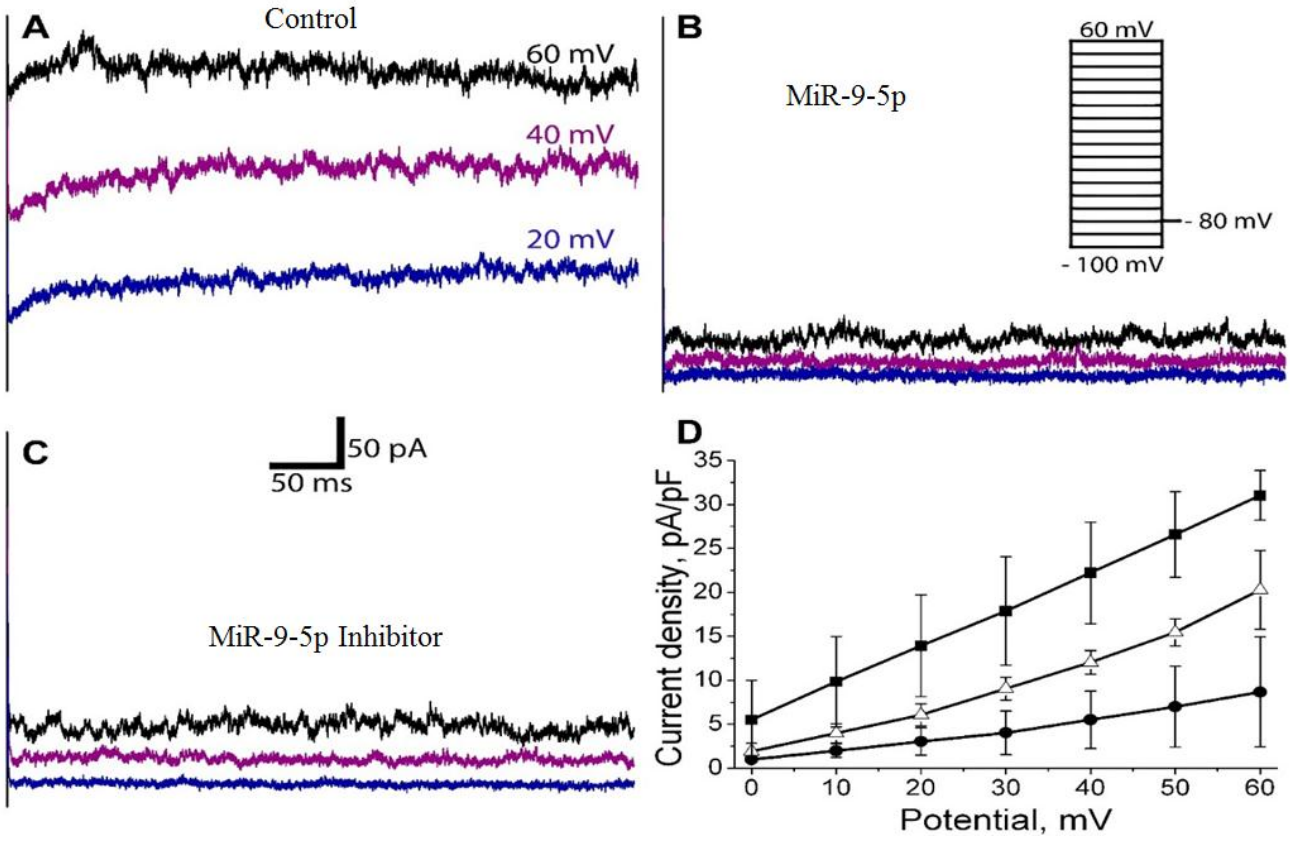

Figure 3. Influence of the MiR-9-5p on the ionic currents through the plasma membrane of osteosarcoma cells (SaOs-2). (A) Control, two components of outward currents are present, one is a rapidly activating current with noisy oscillation, such as Ca^{2+}-activated K^+ current (IK_{Ca}) at potentials +60 mV (black record); and another is a slowly activating current like as delayed rectifier K^+ current (IK_{DR}) at potentials from +20 (blue record) and +40 mV (purple record). **(B)** Cells incubated with MiR-9-5p at the concentration of 30 nM presented reduction in all the ionic currents profile. **(C)** Cells incubated with miR-9-5p inhibitor at the concentration of 30 nM presented partial recovery in all the ionic currents relative to the control. **(D)**; Current-voltage relationship in the control (■), cell incubated with the miR-9-5p (●), or, the miR-9-5p inhibitor (Δ). Membrane currents were elicited by 500 ms voltage steps from a holding potential of –80 to –100 mV up to +60 mV (10 mV increments, sweep interval 2 s) (as shown in the inset of **[B]**).

Supplementary Information

Table S1. MiR-9-5P regulates multiples Ion Channels, Transporters and Receptors

	ION CHANNELS 27		TRANSPORTER 17		RECEPTORS 40
ASIC1	acid-sensing (proton-gated) ion channel 1	SLC10A3	solute carrier family 10 (sodium/bile acid cotransporter family), member 3	PPARA	peroxisome proliferator-activated receptor alpha
CACNB2	calcium channel, voltage-dependent, beta 2 subunit	SLC20A2 (*)	solute carrier family 20 (phosphate transporter), member 2	CNTFR	ciliary neurotrophic factor receptor
CACNA1E (-)	calcium channel, voltage-dependent, R type, alpha 1E subunit	SLC12A5	solute carrier family 12, (potassium-chloride transporter) member 5	PNRC2	proline-rich nuclear receptor coactivator 2
CLCA2	chloride channel accessory 2	SLC27A4	solute carrier family 27 (fatty acid transporter), member 4	LDLRAP1	low density lipoprotein receptor adaptor protein 1
CLCN4	chloride channel, voltage-sensitive 4	SLC30A3	solute carrier family 30 (zinc transporter), member 3	GPR124	G protein-coupled receptor 124
CLCN5	chloride channel, voltage-sensitive 5	SLC16A12	solute carrier family 16, member 12 (monocarboxylic acid transporter 12)	NR2E1	nuclear receptor subfamily 2, group E, member 1
CLCN6	chloride channel, voltage-sensitive 6	SLC19A2	solute carrier family 19 (thiamine transporter), member 2	GIT2	G protein-coupled receptor kinase interactor 2
CLIC4 (*)	chloride intracellular channel 4 Suh 2014	SLC26A2	solute carrier family 26 (sulfate transporter), member 2	TNFRSF21	tumor necrosis factor receptor superfamily, member 21
HCN2	hyperpolarization activated cyclic nucleotide-gated potassium channel 2	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	EPHB4	EPH receptor B4
PIRT	phosphoinositide-interacting regulator of transient receptor potential channels	SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	PTPRK	protein tyrosine phosphatase, receptor type, K
KCTD10	potassium channel tetramerization domain containing 10	SLC39A14	solute carrier family 39 (zinc transporter), member 14	NR5A2	nuclear receptor subfamily 5, group A, member 2
KCTD12	potassium channel tetramerization domain containing 12	ATP8A1	ATPase,aminophospholipid transporter (APLT), class I, type 8A, member 1	GPR137C	G protein-coupled receptor 137C
KCTD2	potassium channel tetramerization domain containing 2	SLC31A2	solute carrier family 31 (copper transporters), member 2	EPHA7	EPH receptor A7
KCTD2	potassium channel tetramerization domain containing 2	SLC5A3	solute carrier family 5 (inositol transporters), member 3	LDLRAD3	low density lipoprotein receptor class A domain containing 3
KCNK10	potassium channel, subfamily K, member 10	SLC7A8	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	DRD2	dopamine receptor D2
KCNK4	potassium channel, subfamily K, member 4	MAGT1	magnesium transporter 1	TGFBR2	transforming growth factor, beta receptor II (70/80kDa)
KCNN3	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	SLC1A1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	GPR123	G protein-coupled receptor 123
KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2			ACVR1C	activin A receptor, type IC
KCNMB2	potassium large conductance calcium-activated channel, subfamily M, beta member 2			GABRB2	gamma-aminobutyric acid (GABA) A receptor, beta 2
KCNQ3 (*)	potassium voltage-gated channel, KQT-like subfamily, member 3. yang 2016			PDGFRB (*)	platelet-derived growth factor receptor, beta polypeptide
KCNB1	potassium voltage-gated channel, Shab-related subfamily, member 1			RIPK5	receptor interacting protein kinase 5
KCNA1	potassium voltage-gated channel, shaker-related subfamily, member 1 (episodic ataxia with myokymia)			LIFR	leukemia inhibitory factor receptor alpha
KCNH5	potassium voltage-gated channel, subfamily H (eag-related), member 5			NCOR2	nuclear receptor co-repressor 2
SCN2B	sodium channel, voltage-gated, type II, beta subunit			NCOA3	nuclear receptor coactivator 3
TRPM7	transient receptor potential cation channel, subfamily M, member 7			RTN4RL1	reticulon 4 receptor-like 1
VDAC3	voltage-dependent anion channel 3			LYVE1	lymphatic vessel endothelial hyaluronan receptor 1
				GRIK3	glutamate receptor, ionotropic, kainate 3

* Genes linked to Primary Familial Brain Calcification

(*) Genes express in SaOs-2 cells

(-) Genes no express in Saos-2 cells

7 CONCLUSÃO

Neste trabalho, investigamos como miR-9-5p, um regulador da expressão gênica, poderia estar envolvido no desenvolvimento ou na potencialização de calcificações, in vitro. Observamos que o miR-9-5p possui sitio de ligação para o *SLC20A2* e *PDGFRB,* regulando-os negativamente. Concluímos que o miR-9-5p é um importante regulador de genes envolvidos na calcificação cerebral familiar primária, durante a calcificação, podendo ser uma via de estudo para entender melhor o processo de calcificação e um potencial alvo terapêutico.

Um modelo, *in vitro*, foi usado para estudar as calcificações e genes associados a calcificações cerebrais primárias familiar (CCPF). Uma linhagem de osteosarcoma (SaOs-2), através de indução química mostrou ser um modelo adequado modelo celular, visto que possui capacidade de calcificação, levando à redução dos principais genes, *SLC20A2* e *PDGFRB,* envolvidos nas CCPF.

REFERÊNCIAS

ALEXIOU, P. et al. Lost in translation: An assessment and perspective for computational microrna target identification. **Bioinformatics**, v. 25, n. 23, p. 3049–3055, 2009.

ARMULIK, A. et al. Pericytes regulate the blood–brain barrier. **Nature**, v. 468, n. 7323, p. 557–561, 25 nov. 2010.

ARPORNMAEKLONG, P. et al. Phenotypic Characterization, Osteoblastic Differentiation, and Bone Regeneration Capacity of Human Embryonic Stem Cell–Derived Mesenchymal Stem Cells. **Stem Cells and Development**, v. 18, n. 7, p. 955–968, 2009.

ARTS, F. A. et al. Idiopathic basal ganglia calcification-associated\n PDGFRB\n mutations impair the receptor signalling. **Journal of Cellular and Molecular Medicine**, v. 19, n. 1, p. 239–248, 2015.

BARBANO, R. et al. Stepwise analysis of MIR9 loci identifies miR-9-5p to be involved in Oestrogen regulated pathways in breast cancer patients. **Scientific Reports**, v. 7, n. March, p. 45283, 2017.

BATLA, A. et al. Deconstructing Fahr's disease/syndrome of brain calcification in the era of new genes. **Parkinsonism & Related Disorders**, v. 37, p. 1–10, abr. 2017.

BERNARDO, B. C. et al. A MicroRNA Guide for Clinicians and Basic Scientists: Background and Experimental Techniques. **Heart Lung and Circulation**, v. 21, n. 3, p. 131–142, 2012.

BIRMINGHAM, E. et al. Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. **European Cells and Materials**, v. 23, n. 353, p. 13–27, 2012.

BLAINE, J.; CHONCHOL, M.; LEVI, M. Renal control of calcium, phosphate, and magnesium homeostasis. **Clinical Journal of the American Society of Nephrology**, v. 10, n. 7, p. 1257–1272, 2015.

BONEV, B.; PISCO, A.; PAPALOPULU, N. MicroRNA-9 Reveals Regional Diversity of Neural Progenitors along the Anterior-Posterior Axis. **Developmental Cell**, v. 20, n. 1, p. 19–32, 2011.

BØTTGER, P. et al. Characterization of transport mechanisms and determinants critical for Na+-dependent Pi symport of the PiT family paralogs human PiT1 and PiT2. **American journal of physiology. Cell physiology**, v. 291, n. 6, p. C1377–C1387, 2006.

CHEN, X. et al. Oncogenic miR-9 is a target of erlotinib in NSCLCs. **Sci Rep**, v. 5, p. 17031, 2015.

COOLEN, M. et al. MiR-9 Controls the Timing of Neurogenesis through the Direct Inhibition of Antagonistic Factors. **Developmental Cell**, v. 22, n. 5, p. 1052–1064, 2012.

COOLEN, M.; KATZ, S.; BALLY-CUIF, L. miR-9: a versatile regulator of neurogenesis. **Frontiers in Cellular Neuroscience**, v. 7, n. November, p. 220, 2013.

DANEMAN, R. et al. Pericytes are required for blood–brain barrier integrity during embryogenesis. **Nature**, v. 468, n. 7323, p. 562–566, 2010.

DANG, C. V. et al. Function of the c-Myc Oncogenic Transcription Factor. **Experimental Cell Research**, v. 253, n. 1, p. 63–77, 1999.

DE OLIVEIRA, J. R. M.; DE OLIVEIRA, M. F. Basal ganglia calcification as a putative cause for cognitive decline. **Dementia & Neuropsychologia**, v. 7, n. 2, p. 151–154, 2013.

DELALOY, C. et al. MicroRNA-9 Coordinates Proliferation and Migration of Human Embryonic Stem Cell-Derived Neural Progenitors. **Cell Stem Cell**, v. 6, n. 4, p. 323–335, abr. 2010.

DRAKAKI, A. et al. Functional microRNA high throughput screening reveals miR-9 as a central regulator of liver oncogenesis by affecting the PPARA-CDH1 pathway. **BMC Cancer**, v. 15, n. 1, p. 542, 2015.

DUMONT, J. et al. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. **Critical Reviews in Biotechnology**, v. 36, n. 6, p.

1110–1122, 2016.

EBERT, M. S.; NEILSON, J. R.; SHARP, P. A. NIH Public Access. v. 4, n. 9, 2013.

FELEKKIS, K. et al. MicroRNAs: A newly described class of encoded molecules that play a role in health and disease. **Hippokratia**, v. 14, n. 4, p. 236–240, 2010.

FERREIRA, D.; ADEGA, F.; CHAVES, R. The Importance of Cancer Cell Lines as in vitro Models in Cancer Methylome Analysis and Anticancer Drugs Testing. **Oncogenomics and Cancer Proteomics- Novel Approaches in Biomarkers Discovery and Therapeutic Targets in Cancer**, p. 139–166, 2013.

GAGLIARDI, M. et al. A SLC20A2 mutation identified in an asymptomatic patient with brain calcification. **Journal of the Neurological Sciences**, v. 372, p. 70–72, 2017.

GE, L. et al. Big Potassium (BK) ion channels in biology, disease and possible targets for cancer immunotherapy. **International Immunopharmacology**, v. 22, n. 2, p. 427–443, out. 2014.

GLASSMEIER, G. et al. Inhibition of HERG1 K + channel protein expression decreases cell proliferation of human small cell lung cancer cells. **Pflugers Archiv European Journal of Physiology**, v. 463, n. 2, p. 365–376, 2012.

HUANG, W. et al. Signaling and transcriptional regulation in osteoblast commitment and differentiation. **Frontiers in bioscience**, v. 12, p. 3068–92, 2007.

INDEN, M. et al. Localization of type-III sodium-dependent phosphate transporter 2 in the mouse brain. **Brain Research**, v. 1531, p. 75–83, 2013.

JANG, S. H. et al. Silencing of Kv4.1 potassium channels inhibits cell proliferation of tumorigenic human mammary epithelial cells. **Biochemical and biophysical research communications**, v. 384, n. 2, p. 180–6, 2009.

JENSEN, N. et al. Loss of function of Slc20a2 associated with familial idiopathic Basal Ganglia calcification in humans causes brain calcifications in mice. **Journal of molecular neuroscience**, v. 51, n. 3, p. 994–9, 2013.

KACZMAREK, L. K. Non-conducting functions of voltage-gated ion channels. **Nature Reviews Neuroscience**, v. 7, n. 10, p. 761–771, 2006.

KAUR, P.; ARMUGAM, A.; JEYASEELAN, K. MicroRNAs in Neurotoxicity. **Journal of toxicology**, v. 2012, p. 870150, 2012.

KEASEY, M. P. et al. Inhibition of a novel specific neuroglial integrin signaling pathway increases STAT3-mediated CNTF expression. **Cell communication and signaling : CCS**, v. 11, n. 1, p. 35, 2013.

KEASEY, M. P. et al. Vitamin-D receptor agonist calcitriol reduces calcification in vitro through selective upregulation of SLC20A2 but not SLC20A1 or XPR1. **Scientific Reports**, v. 6, n. April, p. 25802, 2016.

KELLER, A. et al. Mutations in the gene encoding PDGF-B cause brain calcifications in humans and mice. **Nature genetics**, v. 45, n. 9, p. 1077–82, 2013.

KIM, V. N. MicroRNA biogenesis: coordinated cropping and dicing. **Nature reviews. Molecular cell biology**, v. 6, n. 5, p. 376–85, 2005.

KOZAK, C. A. The mouse "xenotropic" gammaretroviruses and their XPR1 receptor. **Retrovirology**, v. 7, n. 1, p. 101, 2010.

KRICHEVSKY, A. M. et al. Specific MicroRNAs Modulate Embryonic Stem Cell-Derived Neurogenesis. **STEM CELLS**, v. 24, n. 4, p. 857–864, abr. 2006.

KYLE, B. D. et al. regulated by Cysteine String Protein. p. 29–32, 2013.

LANG, F. et al. Ion channels in cell proliferation and apoptotic cell death. **Journal of Membrane Biology**, v. 205, n. 3, p. 147–157, 2005.

LEGATI, A. et al. Mutations in XPR1 cause primary familial brain calcification associated with altered phosphate export. **Nature genetics**, v. 47, n. 6, p. 579–81, 2015a.

LEGATI, A. et al. Mutations in XPR1 cause primary familial brain calcification associated with altered phosphate export. **Nature genetics**, v. 47, n. 6, p. 579–81, 2015b.

LEMOS, R. R. et al. Update and mutational analysis of SLC20A2: A major cause of primary familial brain calcification. **Human Mutation**, v. 36, n. 5, p. 489–495, 2015.

LEUCHT, C. et al. MicroRNA-9 directs late organizer activity of the midbrain-

hindbrain boundary. **Nature Neuroscience**, v. 11, n. 6, p. 641–648, 2008.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and. **Methods**, v. 25, p. 402–408, 2001.

LUKIW, W. J. et al. Common micro RNAs (miRNAs) target complement factor H (CFH) regulation in Alzheimer's disease (AD) and in agerelated macular degeneration (AMD). **International Journal of Biochemistry and Molecular Biology**, v. 3, n. 1, p. 105–116, 2012.

M. WITKOS, T.; KOSCIANSKA, E.; J. KRZYZOSIAK, W. Practical Aspects of microRNA Target Prediction. **Current Molecular Medicine**, v. 11, n. 2, p. 93–109, 2011.

MACK, B. et al. Rapid and Non-Enzymatic In Vitro Retrieval of Tumour Cells from Surgical Specimens. **PLoS ONE**, v. 8, n. 1, p. 1–9, 2013.

MANYAM, B. V. What is and what is not "Fahr"s disease'. **Parkinsonism & Related Disorders**, v. 11, n. 2, p. 73–80, mar. 2005.

MANYAM, B. V.; WALTERS, A. S.; NARLA, K. R. Bilateral Striopallidodentate calcinosis: Clinical characteristics of patients seen in a registry. **Movement Disorders**, v. 16, n. 2, p. 258–264, 2001.

MORI, K. et al. Dexamethasone Enhances In Vitro Vascular Calcification by Promoting Osteoblastic Differentiation of Vascular Smooth Muscle Cells. p. 1–4, 1999.

MOURA, D. A. P.; OLIVEIRA, J. R. M. XPR1: a Gene Linked to Primary Familial Brain Calcification Might Help Explain a Spectrum of Neuropsychiatric Disorders. **Journal of Molecular Neuroscience**, v. 57, n. 4, p. 519–521, 1 dez. 2015.

MUFADDEL, A. A.; AL-HASSANI, G. A. Familial idiopathic basal ganglia calcification (Fahr`s disease). **Neurosciences (Riyadh, Saudi Arabia)**, v. 19, n. 3, p. 171–7, 2014.

NICOLAS, G. et al. Mutation of the PDGFRB gene as a cause of idiopathic basal ganglia calcification. **Neurology**, v. 80, n. 2, p. 181–187, 2013.

NOWEK, K. et al. Expression of a passenger miR-9* predicts favorable outcome in

adults with acute myeloid leukemia less than 60 years of age. **Leukemia**, v. 30, n. 2, p. 303–309, 2016.

OLIVEIRA, J. R. M.; OLIVEIRA, M. F. **Primary brain calcification in patients undergoing treatment with the biphosphanate alendronate.Scientific reports**, 2016. Disponível em: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4792151&tool=pmcentrez&rendertype=abstract>

PAIVA, D. P.; KEASEY, M.; OLIVEIRA, J. R. M. MiR-9-5p Down-Regulates PiT2, but not PiT1 in Human Embryonic Kidney 293 Cells. **Journal of Molecular Neuroscience**, v. 62, n. 1, p. 28–33, 2017.

PAUTKE, C. et al. Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. **Anticancer Research**, v. 24, n. 6, p. 3743–3748, 2004.

PIETRZYKOWSKI, A. Z. et al. Posttranscriptional Regulation of BK Channel Splice Variant Stability by miR-9 Underlies Neuroadaptation to Alcohol. **Neuron**, v. 59, n. 2, p. 274–287, 2008.

PIMENTEL, L. F.; LEMOS, R. R.; OLIVEIRA, J. R. Phosphate Transporters Expression in Patients with Primary Familial Brain Calcifications. **Journal of Molecular Neuroscience**, 2017.

PRESS, D. Fahr ’ s disease : current perspectives. p. 43–49, 2015.

QU, F. et al. MicroRNA‐26a induces osteosarcoma cell growth and metastasis via the Wnt/β‐catenin pathway. **Oncology Letters**, p. 1592–1596, 2015.

RAICA, M.; CIMPEAN, A. M. Platelet-derived growth factor (PDGF)/PDGF receptors (PDGFR) axis as target for antitumor and antiangiogenic therapy. **Pharmaceuticals**, v. 3, n. 3, p. 572–599, 2010.

RAO, V. R. et al. Voltage-gated ion channels in cancer cell proliferation. **Cancers**, v. 7, n. 2, p. 849–875, 2015.

RODAN, S. B. et al. Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties. **Cancer Research**, v. 47, n. 18, p. 4961–4966, 1987.

ROESE-KOERNER, B. et al. Reciprocal Regulation between Bifunctional miR-9/9??? and its Transcriptional Modulator Notch in Human Neural Stem Cell Self-Renewal and Differentiation. **Stem Cell Reports**, v. 7, n. 2, p. 207–219, 2016.

RUPAIMOOLE, R.; SLACK, F. J. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. **Nature Reviews Drug Discovery**, 2017.

SALEEM, S. et al. Fahr's syndrome: literature review of current evidence. **Orphanet journal of rare diseases**, v. 8, n. 1, p. 156, 2013.

SAPIO, L.; NAVIGLIO, S. Inorganic phosphate in the development and treatment of cancer: A Janus Bifrons? **World journal of clinical oncology**, v. 6, n. 6, p. 198–201, 2015.

SHARMA, P. et al. RANKL-RANK signaling regulates expression of xenotropic and polytropic virus receptor (XPR1) in osteoclasts. **Biochemical and Biophysical Research Communications**, v. 399, n. 2, p. 129–132, 2010.

SHIEH, C.-C. et al. Potassium Channels: Molecular Defects, Diseases, and Therapeutic Opportunities. **Pharmacol. Rev.**, v. 52, n. 4, p. 557–594, 2000.

SILA-ASNA, M. et al. Osteoblast differentiation and bone formation gene expression in strontium-inducing bone marrow mesenchymal stem cell. **The Kobe journal of medical sciences**, v. 53, n. 1–2, p. 25–35, 2007.

SIOMI, H.; SIOMI, M. C. Posttranscriptional Regulation of MicroRNA Biogenesis in Animals. **Molecular Cell**, v. 38, n. 3, p. 323–332, 14 maio 2010.

SOBRIDO, M. J. et al. Primary Familial Brain Calcification. **GeneReviews(®)**, p. 1–19, 2014.

SORIANO, P. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. **Genes & Development**, v. 8, n. 16, p. 1888–1896, 1994.

STAPPERT, L.; ROESE-KOERNER, B.; BRÜSTLE, O. The role of microRNAs in human neural stem cells, neuronal differentiation and subtype specification. **Cell and Tissue Research**, v. 359, n. 1, p. 47–64, 2015.

STENVANG, J. et al. Inhibition of microRNA function by antimiR oligonucleotides.

Silence, v. 3, n. 1, p. 1–17, 2012.

SUN, L.-H. et al. MicroRNA-9 induces defective trafficking of Nav1.1 and Nav1.2 by targeting Navβ2 protein coding region in rat with chronic brain hypoperfusion. **Molecular Neurodegeneration**, v. 10, n. 1, p. 36, 2015.

TADIC, V. et al. Primary Familial Brain Calcification With Known Gene Mutations. **JAMA Neurology**, v. 72, n. 4, p. 460, 2015.

TAGLIA, I. et al. Primary familial brain calcification: update on molecular genetics. **Neurological Sciences**, v. 36, n. 5, p. 787–794, 2015.

TO, C. Gastrointestinal Absorption of Heparins. n. November, p. 1–22, 2009.

TOPOL, A. et al. Dysregulation of miRNA-9 in a Subset of Schizophrenia Patient-Derived Neural Progenitor Cells. **Cell Reports**, v. 15, n. 5, p. 1024–1036, 2016.

URREGO, D. et al. Potassium channels in cell cycle and cell proliferation. **Philosophical transactions of the Royal Society of London. Series B, Biological sciences**, v. 369, n. 1638, p. 20130094, 2014.

WAHID, F. et al. MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. **Biochimica et Biophysica Acta - Molecular Cell Research**, v. 1803, n. 11, p. 1231–1243, 2010.

WALLINGFORD, M. C. et al. SLC20A2 Deficiency in Mice Leads to Elevated Phosphate Levels in Cerbrospinal Fluid and Glymphatic Pathway-Associated Arteriolar Calcification, and Recapitulates Human Idiopathic Basal Ganglia Calcification. **Brain Pathology**, v. 3, n. 28, p. 64–76, 2016.

WALLINGFORD, M. C.; GAMMILL, H. S.; GIACHELLI, C. M. Slc20a2 deficiency results in fetal growth restriction and placental calcification associated with thickened basement membranes and novel CD13 and lamininα1 expressing cells. **Reproductive Biology**, v. 16, n. 1, p. 13–26, 2016.

WANG, C. et al. Mutations in SLC20A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. **Nature genetics**, v. 44, n. 3, p. 254–6, 2012.

WANG, Y.; BLELLOCH, R. NIH Public Access. **Cancer**, v. 69, n. 10, p. 4093–4096,

2010.

WANG, Z. H. et al. Blockage of intermediate-conductance-Ca2+-activated K+ channels inhibits progression of human endometrial cancer. **Oncogene**, v. 26, n. 35, p. 5107–5114, 2007.

WIDER, C. et al. Familial idiopathic basal ganglia calcification: A challenging clinical-pathological correlation. **Journal of Neurology**, v. 256, n. 5, p. 839–842, 2009.

WINTER, J. et al. Many roads to maturity: microRNA biogenesis pathways and their regulation. **Nature cell biology**, v. 11, n. 3, p. 228–234, 2009.

YANG, L. et al. Involvement of miR-9/MCPIP1 axis in PDGF-BB-mediated neurogenesis in neuronal progenitor cells. **Cell death & disease**, v. 4, n. 12, p. e960, 2013.

YASUDA, T.; CUNY, H.; ADAMS, D. J. K_v 3.1 channels stimulate adult neural precursor cell proliferation and neuronal differentiation. **The Journal of Physiology**, v. 591, n. 10, p. 2579–2591, 2013.

YUVA-AYDEMIR, Y. et al. MicroRNA-9. **RNA Biology**, v. 8, n. 4, p. 557–564, 27 jul. 2011.

ZAMORE, P. D. A Micr oRNA in a Multiple-Tur nover RNAi Enzyme Complex. n. 3, p. 1–8, [s.d.].

ZAWISTOWSKI, J. S. et al. MicroRNA 9-3p Targets 1 Integrin To Sensitize Claudin-Low Breast Cancer Cells to MEK Inhibition. **Molecular and Cellular Biology**, v. 33, n. 11, p. 2260–2274, 2013.

ZHANG, H.; SHYKIND, B.; SUN, T. Approaches to manipulating micrornas in neurogenesis. **Frontiers in Neuroscience**, v. 6, n. January, p. 1–13, 2012.

ZHANG, J. et al. MicroRNA-9 is an activation-induced regulator of PDGFR-beta expression in cardiomyocytes. **Journal of Molecular and Cellular Cardiology**, v. 51, n. 3, p. 409–418, 2011.

ZIMMERMAN, A. L.; WU, S. MicroRNAs, cancer and cancer stem cells. **Cancer Letters**, v. 300, n. 1, p. 10–19, jan. 2011.

ANEXO A - APRESENTAÇÃO DE PÔSTER NO SINATER

SINATER
VI Simpósio Internacional em Diagnóstico e Terapêutica

Recife, 14 de janeiro de 2016

CERTIFICADO

Certifico que **Darlene Paiva Bezerra** apresentou na forma de pôster o trabalho intitulado: **Influence of microRNA9 in the development or enhancement of calcification in vitro** de autoria de Darlene Paiva Bezerra e João Ricardo Mendes de Oliveira, no VI SINATER - Simpósio Internacional em Diagnóstico e Terapêutica e IX Jornada Científica do LIKA realizado de 18 a 21 de agosto de 2015 no auditório do Centro de Pesquisas Aggeu Magalhães.

Atenciosamente,

ANEXO B - NORMAS DO PERIÓDICO SCIENTIFIC REPORT

General information for preparing manuscripts

Format of articles

Scientific Reports publishes original research in one format, Article. In most cases we do not impose strict limits on word count or page number. We do, however, strongly encourage authors to write concisely and to adhere to the guidelines below.

Articles should ideally be no more than 11 typeset pages in length. As a guide, the main text (not including Abstract, Methods, References and figure legends) should be no more than 4,500 words. The maximum Article title length is 20 words. The Abstract — which must be no more than 200 words long and contain no references — should serve both as a general introduction to the topic and as a brief, non-technical summary of the main results and their implications.

For the main body of the text, there are no explicit requirements for section organization. According to the authors' preference, the text may be organized as best suits the research. As a guideline and in the majority of cases, however, we recommend that you structure your manuscript as follows:

Introduction

Results (with subheadings)

Discussion (without subheadings)

Methods

A specific order for the main body of the text is not compulsory and, in some cases, it may be appropriate to combine sections. Figure legends are limited to 350 words. As a guideline references should be limited to 60 (this is not strictly enforced). Footnotes should not be used.

We suggest that Articles contain no more than 8 display items (figures and/or tables). In addition, a limited number of uncaptioned molecular structure graphics and numbered mathematical equations may be included if necessary. To enable typesetting of papers, the number of display items should be commensurate with the word length — we suggest that for Articles with less than 2,000 words, no more than 4 figures/tables should be included. Please note that schemes are not used and should be presented as figures.

Authors must provide a competing financial interests statement within the manuscript file.

Submissions should include a cover letter, a manuscript text file, individual figure files and optional supplementary information files. For first submissions (i.e. not revised manuscripts), authors may incorporate the manuscript text and figures into a single file up to 3 MB in size; the figures may be inserted in the text at the appropriate positions, or grouped at the end. Supplementary information should be combined and supplied as a single separate file, preferably in PDF format.

ONLY the following file types can be uploaded for Article text:

txt, doc, docx, tex

A submission template is available in the Overleaf template gallery to help you prepare a LaTeX manuscript within the Scientific Reports formatting criteria.

Scientific Reports is read by scientists from diverse backgrounds. In addition, many are not native English speakers. Authors should, therefore, give careful thought to how their findings may be communicated clearly. Although a shared basic knowledge of science may be assumed, please bear in mind that the language and concepts that are standard in one field may be unfamiliar to non-specialists. Thus, technical jargon should be avoided and clearly explained where its use is unavoidable.

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Format of manuscripts

In most cases we do not impose strict limits on word counts and page numbers, but we encourage authors to write concisely and suggest authors adhere to the guidelines below. For a definitive list of which limits are mandatory please visit the submission checklist page.

Articles should be no more than 11 typeset pages in length. As a guide, the main text (not including Abstract, Methods, References and figure legends) should be no more than 4,500 words. The maximum title length is 20 words. The Abstract (without heading) - which must be no more than 200 words long and contain no references - should serve both as a general introduction to the topic and as a brief, non-technical summary of the main results and their implications.

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Smith, J. Syntax of referencing in How to reference books (ed. Smith, S.) 180-181 (Macmillan, 2013).

Online material:

Manaster, J. Sloth squeak. Scientific American Blog Network http://blogs.scientificamerican.com/psi-vid/2014/04/09/sloth-squeak (2014).

Hao, Z., AghaKouchak, A., Nakhjiri, N. & Farahmand, A. Global integrated drought monitoring and prediction system (GIDMaPS) data sets. figshare http://dx.doi.org/10.6084/m9.figshare.853801 (2014).

Acknowledgements

Acknowledgements should be brief, and should not include thanks to anonymous referees and editors, or effusive comments. Grant or contribution numbers may be acknowledged. Assistance from medical writers, proof-readers and editors should also be acknowledged here.

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