UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE: CARDIOLOGIA E CIÊNCIAS CARDIOVASCULARES

Tese

MicroRNAs e vias de sinalização na hipertrofia cardíaca fisiológica.

Graziela Hünning Pinto

Análise do perfil de miRNAs e vias de sinalização relacionadas em modelo animal de hipertrofia cardíaca fisiológica induzida por natação.

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Ago2: Argonaut 2

ANG II: Angiotensina II

AT1: Receptor AT1

AT2: Receptor AT2

FGF: Fibroblast Growth Factor

HAS: Hipertensão Arterial Sistêmica

HC: Hipertrofia Cardíaca

HCF: Hipertrofia Cardíaca Fisiológica

HCP: Hipertrofia Cardíaca Patológica

HDL: Lipoproteína de alta densidade

IAM: Infarto Agudo do Miocárdio

IC: Insuficiência Cardíaca

miRNA ou miR: microRNA

MHC-β – Miosina de Cadeia Pesada Beta

MSTN: miostatina

mTOR: Proteína quinase alvo da rapamicina

PCR: reação em cadeia da polimerase

PE: fosfatidiletanolamina

PI3K: Fosfatildilinositol-4,5-bifostato 3 Quinase

PKB ou AKT: Proteina quinase B

TNF-α: Tumor Necrosis Factor

TGF-β: Transforming Growth Factor β

THRAP1: Receptor do Hormônio da Tireóide

VE: ventrículo esquerdo

VEGF: Fator de Crescimento Endotelial Vascular

APRESENTAÇÃO

A presente tese de doutorado será apresentada na seguinte disposição:

Parte I: Resumo, Introdução, Justificativa, Hipótese, Objetivos, Referências

Parte II: Artigo 1

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Parte I

Resumo

A hipertrofia cardíaca é um evento decorrente da adaptação do tecido cardíaco frente a estímulos de crescimento muscular. O caráter fisiológico que ocorre de forma reversível podemos exemplificar pelo evento da gravidez ou exercício físico. Por outro lado, o perfil patológico apresenta característica irreversível como por exemplo a sobrecarga de pressão causada pela hipertensão arterial sistêmica. De acordo com o tipo de estímulo recebido pelo coração determinadas alterações bioquímicas e moleculares podem responder de forma a desenvolver a hipertrofia do tipo fisiológica ou patológica. Os microRNAS (miRNAs) são moléculas endógenas, pequenos RNAs altamente conservados que, geralmente, não codificam proteínas e que podem apresentar expressão aumentada ou expressão reduzida na hipertrofia cardíaca. A literatura aborda especialmente o perfil de expressão dos miRNAs na hipertrofia cardíaca patológica, contudo no modelo fisiológico poucos autores têm avaliado e revisado. O objetivo deste trabalho foi avaliar as alterações na expressão de miRNAs associadas a modelos de hipertrofia cardíaca fisiológica. A revisão sistemática identificou 11 estudos os quais foram incluídos para a análise final. Dentre os estudos selecionados apenas estudos experimentais foram incluídos de acordo com os critérios de seleção. A maioria dos estudos selecionados é oriunda de pesquisas brasileiras e desenvolveram protocolo de hipertrofia por natação. Dentre os miRNAs identificados nos estudos incluídos, 29 miRNAs apresentaram expressão aumentada enquanto 14 apresentaram expressão reduzida. Cerca de 5 miRNAs apresentaram-se aumentados em alguns estudos enquanto que em outros estudos apresentaram expressão reduzida. Alguns

miRNAs avaliados por mais de um autor apresentaram expressão de forma divergente (miR-21, miR-27a e miR-222) entre os estudos em diferente espécies. Por outro lado,foi possível verificar estudos que concordaram quanto a expressão de determinados miRNAs inclusive em diferentes espécies (miR-1, miR-143 e miR499). . O perfil de expressão dos miRNAs -1, -143 e -499 parece ser consistente em mais de um estudo. Contudo, existe grande variabilidade de modelos e de resultados em relação a hipertrofia cardíaca fisiológica e expressão de miRNAs. No estudo experimental demonstramos a hipertrofia cardíaca fisiológica após 7 e 28 dias de natação. Os miRNAs diferencialmente expressos foram identificados por microarranjo (9 miRNAs com expressão reduzida e 13 com expressão reduzida). Os miRNAs escolhidos que apresentaram-se como diferencialmente expressos foram validados por outra técnica, PCR em tempo real, na amostra total. Os miRNAs -10a-5p, -29c-5p, -212-5p, -21a-5p, -206-3p, -34b-3p e -215-5p apresentaram-se aumentados no grupo treinado 28 dias, enquanto que o miR-329-3p apresentou aumento em ambos os grupos treinados. A análise de bioinformática identificou possíveis alvos e vias envolvidas com os miRNAs validados. Cerca de 930 genes foram relacionados com miRNAs que apresentaram expressão aumentada e reduzida. Também, a análise de enriquecimento funcional trouxe 12 vias relacionadas aos miRNAs com expressão reduzida e 92 com expressão aumentada. A maioria das vias envolvidas com os miRNAs aumentados estão relacionadas com crescimento e sobrevivência celular, contudo foi destacada a via da MAPK e da insulina como vias mais importantes. O miR-212-5p e o -329-3p se destacaram devido possíveis genes alvos preditos e não validades que se relacionaram com as vias MAPK e insulina escolhidas como objeto de

estudo. Além disso, o miR-329-3p foi o miRNA com maior quantidade de alvos preditos dentro das vias MAPK e insulina. Dessa forma, considerando os resultados que encontramos na análise do perfil de expressão dos miRNAs podemos concluir que a expressão aumentada de alguns miRNAs em modelo animal de hipertrofia cardíaca fisiológica parece estar relacionada a via da MAPK e insulina, além da participação de genes envolvidos no crescimento celular. Também, o miR-212-5p e o -329-3p merecem destaque devido poucas evidências e sua importância nesse fenótipo.

1. Referencial Teórico

1.1 Hipertrofia Cardíaca

A hipertrofia cardíaca (HC) se caracteriza pelo aumento da massa do coração, devido a um aumento nas dimensões dos cardiomiócitos. Este processo provoca aumento no consumo de oxigênio e nutrientes nos cardiomiócitos e, como consequência, promove mudanças funcionais e bioquímicas nas células (ANVERSA; RICCI; OLIVETTI, 1986; GUPTA, 2007; MARON; PELLICCIA, 2006). De acordo com o tipo de estímulo recebido pelo tecido cardíaco as alterações estruturais e bioquímicas dos cardiomiócitos podem determinar se a hipertrofia presente no tecido é de natureza fisiológica ou patológica (BRAUNWALD; BRISTOW, 2000; IEMITSU et al., 2001).

A HC fisiológica demonstra um aumento dos cardiomiócitos em resposta ao exercício, e os cardiomiócitos aumentados recebem nutrição adequada devido à expansão da rede capilar. Anormalidades cardíacas estruturais ou funcionais não ocorrem nesse cenário, e, geralmente, a HC fisiológica não é considerada um fator de risco para insuficiência cardíaca. Os estímulos fisiológicos podem ocorrer de forma concêntrica ou excêntrica, no entanto, o alargamento das câmaras se desenvolve de uma forma proporcional, desencadeando uma melhora da função cardíaca sem a presença de fibrose conforme demonstra a Figura 1 (BERNARDO et al., 2010).

Por outro lado, a HC patológica está associada aos altos níveis de mediadores neuro-humorais, sobrecarga hemodinâmica, lesão e perda de cardiomiócitos. No desenvolvimento de uma HC em cenário patológico, o crescimento de cardiomiócitos excede a capacidade dos capilares de fornecer adequadamente nutrientes e oxigênio, levando à hipóxia e remodelamento cardíaco em roedores (SANO et al., 2007;

SHIMIZU et al., 2010). A sobrecarga pressórica promove um crescimento concêntrico, enquanto que uma sobrecarga de volume desenvolverá um crescimento excêntrico (FOPPA; DUNCAN; ROHDE, 2005). No modelo patológico há perda funcional do tecido cardíaco devido à morte celular, além da presença de fibrose no tecido cardíaco, o que pode originar a insuficiência cardíaca (SHIMIZU; MINAMINO, 2016). Pesquisadores discutem que a hipertrofia cardíaca é uma das causas de morbidade e mortalidade por doenças cardiovasculares mais comuns. Tanto na hipertensão arterial sistêmica assim como nas cardiomiopatias, a presença de hipertrofia cardíaca constitui fator de risco para morte súbita. Durante o estágio compensatório da hipertrofia, o aumento do tamanho e da massa do coração é acompanhado por alterações bioquímicas, moleculares, estruturais e metabólicas, a fim de manter a função cardíaca. Com o tempo, no entanto, o estresse crônico ou doença resultará em dilatação ventricular, queda na função contrátil e, eventualmente, progredirá para insuficiência cardíaca (THAM et al., 2015).

A insuficiência cardíaca é uma doença que atinge de forma global cerca de 38 milhões de pessoas no mundo segundo Braunwald e colaboradores (BRAUNWALD; BRISTOW, 2000). Em 2014 pesquisadores relataram que entre 1990 e 2010 a doença cardíaca isquêmica foi a causa mais comum de morte no mundo. Embora a incidência padronizada por idade do infarto agudo do miocárdio (IAM) tenha reduzido em todo o mundo, a prevalência de insuficiência cardíaca aumentou (MORAN et al., 2014).

1.2 Hipertrofia Cardíaca Patológica

Diversas condições patológicas se associam à HC patológica. A hipertensão arterial sistêmica, por exemplo, é uma condição hemodinâmica de caráter não transitório que exerce grande estresse às paredes cardíacas causando uma sobrecarga pressórica (BERNARDO et al., 2010; KEMPF; WOLLERT, 2004). Em resposta a esta condição, os cardiomiócitos aumentam a síntese de sarcômeros em paralelo, característico da hipertrofia concêntrica, e não há biogênese de novos componentes celulares (BERNARDO et al., 2010; KACIMI; GERDES, 2003). Outra condição hemodinâmica importante é a sobrecarga de volume dentro das cavidades cardíacas. Neste caso, as fibras musculares estão distendidas nos sarcômeros ao máximo, o que prejudica a contração do miocárdio. A fim de responder a este evento há síntese de sarcômeros em série, característico da hipertrofia excêntrica conforme pesquisadores revisaram recentemente sendo exemplificado na figura 1 (BERNARDO et al., 2010; SHIMIZU; MINAMINO, 2016).

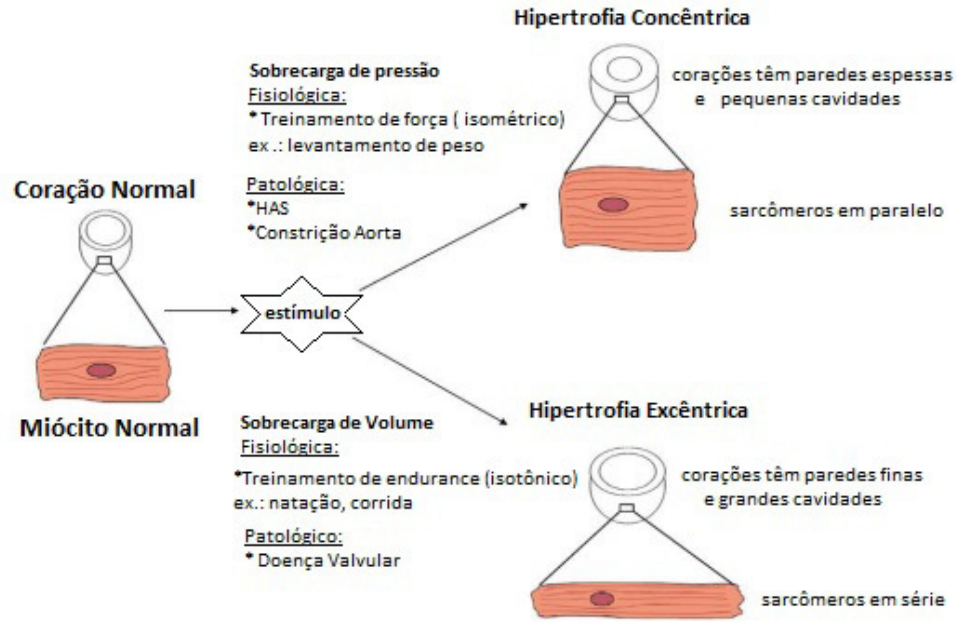

Figura 1. Estímulo Hipertrófico na HCF e HCP adaptado (BERNARDO et al., 2010).

Os mecanismos pelos quais o miocárdio inicia o processo de hipertrofia ainda são pouco conhecidos. Sabe-se que diversos sistemas participam desta ação (sistema simpático, renina-angiotensina-aldosterona e endotelina) por meio de diversos mediadores inflamatórios como o fator de necrose tumoral (TNF-α), fator de crescimento transformante β1 (TGFβ1), fator de crescimento de fibroblastos 2 (FGF2), proteína G e moléculas produzidas durante o estresse oxidativo (HEINEKE; MOLKENTIN, 2006; SHIMIZU; MINAMINO, 2016).

O sistema renina-angiotensina (SRA) pode ser induzido de 2 formas: (i) ativação sistêmica (baixa eliminação de cloreto e aumento de estímulo beta adrenérgico) (SHAH et al., 2001); ou (ii) por estiramento mecânico (produção local de angiotensina II) (SADOSHIMA et al., 1993) . A produção autócrina de ANGII ocorre devido ao recrutamento de células inflamatórias capazes de expressar a enzima conversora de angiotensina (ECA), responsável pela síntese local de ANGII (SUTTON; ST. J.

SUTTON; SHARPE, 2000). Uma vez produzida, a ANGII pode ligar-se ao seu receptor específico AT1 promovendo uma série de respostas associadas aos processos hipertrófico, fibrótico e apoptótico desencadeados por ação de citocinas inflamatórias (BRAUNWALD; BRISTOW, 2000; TAMURA et al., 2000). A exemplo de citocinas podemos citar o fator nuclear kappa da cadeia de células B ativadas (NF-kB) o qual é um dos reguladores críticos da inflamação e conhecido por aumentar a produção de citocinas pró-inflamatórias, como o TNF-α. Recentemente, foi relatado que o NF-kB medeia a hipertrofia de cardiomiócitos induzida pela angiotensina II e pela endotelina-1 (RAJAPUROHITAM et al., 2012), mas os dados obtidos a partir de camundongos transgênicos são conflitantes. Em um modelo de sobrecarga de pressão murina, a deleção de p65 NF-κB nos cardiomiócitos reduziu a função cardíaca juntamente com a diminuição da vascularização e aumento da fibrose (JAVAN et al., 2015). Contudo, outro estudo mostrou que a sinalização de NF-κB/Fator nuclear de calcineurina de células T ativadas (NFAT) promoveria hipertrofia cardíaca e remodelação ventricular (LIU et al., 2012).

Além disso, a interleucina 10 (IL-10), uma citocina anti-inflamatória, demonstrou suprimir a hipertrofia cardíaca induzida por sobrecarga de pressão do ventrículo esquerdo (VE) e inibição da sinalização de NF-κB (VERMA et al., 2012). Curiosamente, no modelo de sobrecarga de pressão de VE, o acúmulo de células T mostrou contribuir para um aumento na expressão de IL-10 no tecido cardíaco (YANG et al., 2012). Estudos indicam que, de acordo com o tipo de estímulo hipertrófico, as comunicações intercelulares são mediadas por citocinas pró ou anti-inflamatórias secretadas por cardiomiócitos ou outras células os quais participam da hipertrofia cardíaca (FRIELER;

MORTENSEN, 2015; GHIGO et al., 2014; KAMO; AKAZAWA; KOMURO, 2015). Além das citocinas, outros fatores endógenos podem contribuir para hipertrofia cardíaca como o TNF-α e outros peptídeos os quais desencadeiam a desregulação de vias como a autofagia, modificação de DNA e histonas, mudanças na expressão de miRNAs e metabolismo em cardiomiócitos. O TNF-α secretado por cardiomiócitos induz hipertrofia cardíaca, ao passo que o peptídeo natriurético atrial (ANP), o peptídeo natriurético cerebral (BNP) e a adrenomedulina inibem essa resposta de maneira autócrina conforme autores revisaram recentemente (Figura 2A) (SHIMIZU; MINAMINO, 2016).

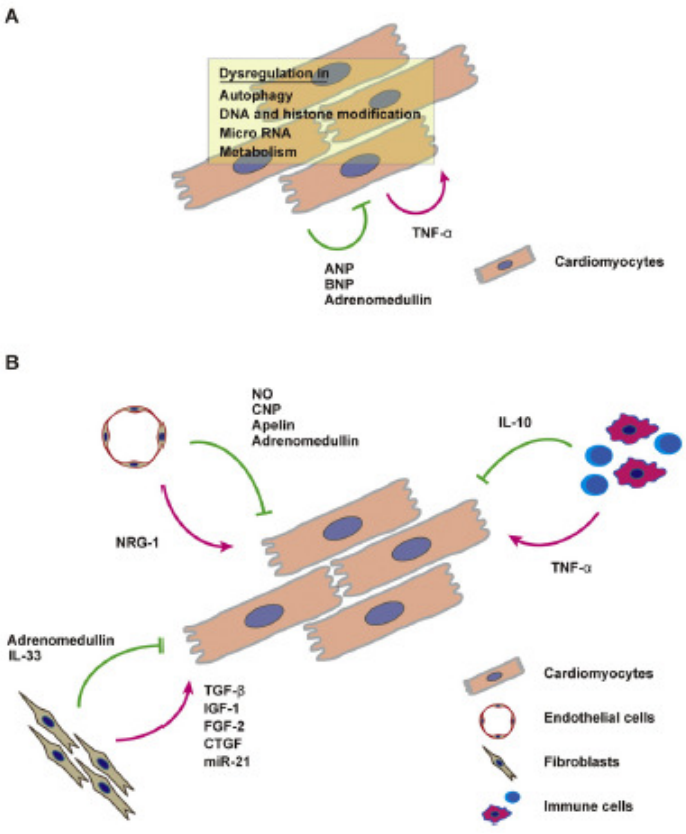

Figura 2. Modificadores da hipertrofia cardíaca. A. Fatores endógenos que contribuem para a indução ou supressão da hipertrofia cardíaca. B. Fatores exógenos que contribuem para a indução ou supressão da hipertrofia cardíaca (SHIMIZU; MINAMINO, 2016).

Quanto aos fatores exógenos, sabe-se que as células endoteliais secretam mediadores anti-hipertróficos como óxido nítrico (NO), peptídeo natriurético do tipo C (CNP), apelina e adrenomedulina. As células endoteliais também secretam um fator pró-hipertrófico chamado neuregulina-1 (NRG-1). Por outro lado, os fibroblastos secretam moléculas predominantemente pró-hipertróficas como TGF-β, fator de crescimento semelhante à insulina 1 (IGF-1), fator de crescimento de fibroblastos 2 (FGF-2), fator de crescimento do tecido conjuntivo (CTGF) e inclusive pequenos RNAs como o miRNA-21. Os fibroblastos também secretam moléculas anti-hipertróficas como adrenomedulina e interleucina-33 (IL-33) com a finalidade de inibir a hipertrofia. Adicionalmente, as células imunológicas também contribuem para respostas pró ou anti-hipertróficas, e estas são mediadas por TNF-α ou interleucina-10 (IL-10), respectivamente (Figura 2B) (SHIMIZU; MINAMINO, 2016).

Dessa forma, a HC patológica se desenvolve frente a sinalizadores moleculares e bioquímicos. Em uma resposta a neuro-hormônios e estímulos mecânicos fisiológicos, o crescimento dos miócitos na HC fisiológica também sofre influência da ativação de sinalizadores presentes em vias relacionadas à sobrevivência celular o que contribui para o desenvolvimento da massa cardíaca sem a presença de fibrose (LYON et al., 2015) .

1.3 Hipertrofia Cardíaca Fisiológica

O aumento do coração em resposta ao treinamento físico e a gravidez são eventos transitórios atribuídos à HC fisiológica. O aumento do peso corporal e do peso

do ventrículo durante o crescimento da massa cardíaca é atribuído ao aumento dos cardiomiócitos em que há aumento de quase 3 vezes do diâmetro dos cardiomiócitos ao longo do desenvolvimento de lactentes. Tanto o tamanho quanto a função cardíaca são dependentes da angiogênese e a desregulação da coordenação entre crescimento dos cardiomiócitos e a angiogênese no coração tem um papel causal na mudança da hipertrofia cardíaca adaptativa para insuficiência cardíaca (SANO et al., 2007; SHIMIZU et al., 2010; SHIOJIMA et al., 2005). A hipertrofia do ventrículo esquerdo ocorre por um aumento uniforme da parede ventricular sem o desenvolvimento de fibrose e/ou disfunção cardíaca. No entanto, com o cessar do estímulo, o coração volta às dimensões originais, sendo esta a hipertrofia adaptativa (fisiológica) (FERNANDES; SOCI; OLIVEIRA, 2011)

Na hipertrofia fisiológica o crescimento do músculo cardíaco pode ser do tipo concêntrico ou excêntrico dependendo do tipo de estímulo realizado. O exercício físico isométrico desenvolvido, por exemplo, por um treinamento de resistência leva à sobrecarga pressórica, o que caracteriza a hipertrofia concêntrica, devido à adição de sarcômeros em paralelo apresentando paredes espessas e cavidades pequenas. Já o exercício isotônico leva a sobrecarga de volume, promove uma hipertrofia excêntrica apresentando sarcômeros adicionados em série o que resulta em aumento de cavidades cardíacas e menor aumento de espessuras parietais (BERNARDO et al., 2010). Evangelista e colaboradores demonstraram estes eventos em modelo animal de HC fisiológica, no qual camundongos foram submetidos a duas sessões de 90 min de natação, 5 dias da semana durante 4 semanas, e o grupo treinado apresentou maior índice de hipertrofia ventricular que o grupo sedentário (EVANGELISTA; BRUM;

KRIEGER, 2003). Diferenças entre a hipertrofia induzida por sobrecarga pressórica e de volume estão exemplificados na figura 1.

Diferentes protocolos de treinamento físico, os quais induzem a HC seja por sobrecarga de pressão ou por sobrecarga de volume, desenvolvem diferentes fenótipos de hipertrofia cardíaca conforme revisou Fernandes e colaboradores e podemos observar na figura 3. Exercícios aeróbicos, como correr ou nadar, facilitam o retorno venoso e aumentam o volume diastólico final, uma vez que há contração muscular realizada por longos períodos (30-60 min) além de demandar o oxigênio para os músculos ativos (FERNANDES et al., 2015) . Por outro lado, exercícios de resistência ou força, como levantamento de peso, demandam em menor proporção a massa muscular, mas a contração de força é limitada a poucas repetições (geralmente, 20) até a exaustão o que aumenta a resistência vascular sistêmica. Portanto, a magnitude da hipertrofia cardíaca é muito menor em resposta aos exercícios de resistência / força do que os exercícios aeróbicos (SPENCE et al., 2011).

Os mecanismos moleculares responsáveis pelo aumento cardíaco causado pelo exercício ainda estão sendo explorados. O fenótipo molecular fisiológico difere do encontrado nas doenças cardíacas. Durante o exercício, a participação local da angiotensina II (AGII) parece ocorrer por meio do receptor AT2 que atua neutralizando efeitos do receptor AT1 representando uma proteção ao coração (DOSTAL, 2000; WAGENAAR et al., 2002). Camundongos knockout para os receptores adrenérgicos (α_{2A}/α_{2C} ARKO) desenvolvem insuficiência cardíaca devido a um aumento nos níveis de ANGII tecidual. Adicionalmente, neste modelo animal, o exercício físico foi capaz de atenuar o processo de remodelamento ao diminuir a atividade da enzima conversora de

angiotensina tecidual (ECA_t), assim como reduzir os níveis de AGII. No entanto, a expressão de AT1 não mudou nos animais exercitados. Estes resultados sugerem que a ação do AT2 possa estar prevenindo o processo hipertrófico patológico (PEREIRA et al., 2009).

As vias de sobrevivência celular estão intimamente relacionadas ao processo de hipertrofia. O estiramento mecânico causado pelo exercício ativa a proteína quinase B (PKB) ou AKT o qual fosforila diversos substratos intracelulares responsáveis pela regulação do crescimento, metabolismo e sobrevivência celular (ZHAI, 2006). No estado ativado a AKT promove ativação da proteína quinase mTOR (proteína alvo da rapamicina em mamíferos) aumentando a síntese proteica (PROUD, 2004). Portanto, quando mTOR está ativada há progressão da síntese proteica e hipertrofia. A mTOR é uma grande proteína quinase serina-treonina que pertence à família relacionada à fosfatidilinositol-3 quinase (PI3K) (YANG; GUAN, 2007). A fosfatidilinositol 3-quinase (PI3K) catalisa a fosforilação de lipídios de membrana, conhecido como fosfoinositídeos e, assim, ativa uma série de moléculas de sinalização intracelular, como AKT1, que é um efetor principal a jusante de PI3K. A AKT1 é fosforilada em hipertrofia cardíaca fisiológica e exerce diversas funções benéficas, como inibição da apoptose de cardiomiócitos, melhora dos transientes de cálcio e hipertrofia cardíaca (KIM et al., 2012).

Sendo assim, a mTOR tem como função promover o crescimento e proliferação celular, responder a sinais da AKT, além de controlar processos celulares incluindo autofagia (HAY, 2004). No músculo cardíaco exercitado a cascata AKT/mTOR induz crescimento de cardiomiócito melhorando força contrátil e hipertrofia ventricular. Dessa

forma, o controle do crescimento muscular ocorre pela via IGF-PI3KAKT/mTOR, sendo este um regulador positivo da hipertrofia cardíaca fisiológica (MCMULLEN et al., 2003, 2004; WEEKS; MCMULLEN, 2011). Recente estudo de nosso grupo avaliou a HC fisiológica induzida por natação em camundongos o qual apresentou expressão aumentada de mTOR em tecido cardíaco no grupo treinado, corroborando esse achado com estudos prévios (PINTO, 2014).

O exercício físico também modula outras moléculas biológicas envolvidas no crescimento e sobrevivência dos cardiomiócitos. Os miRNAs podem regular processos de anti-fibrose como exemplificado pelo miR-29a e miR-29c, assim como o miR-216 modula a angiogênese em resposta ao treinamento aeróbico. Há um grande número de miRNAs participando do crescimento e sobrevivência do músculo cardíaco como por exemplo miR-1, -21, -26b, -27a / b, -30e, -99b, -100, -124, -133a / b, -143, -144, -145, -150, -208a e -222 os quais podem apresentar expressões diferenciadas após o estímulo hipertrófico causado pelo exercício conforme pesquisadores revisaram recentemente e podemos observar exemplificado na figura 3 (FERNANDES et al., 2015).

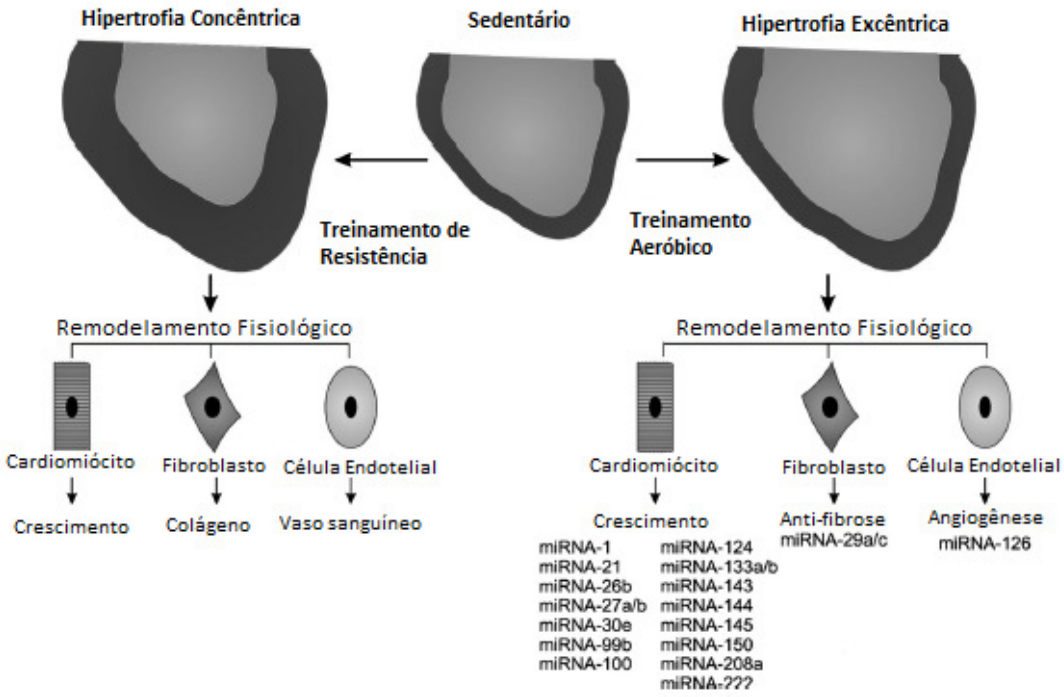

Figura 3. Esquema de remodelamento cardíaco fisiológico induzido por treinamento físico adaptado de Fernandes e colaboradores (FERNANDES et al., 2015)**.**

1.4 MiRNAs

Os microRNAs (miRNAs ou miRs) são pequenos RNAs altamente conservados (19-25 nucleotídeos) que, geralmente, não codificam proteínas. Entretanto, os miRNAs são produzidos endogenamente e cuja função é regular a expressão gênica diminuindo a expressão do gene ou proteína pós-transcricionalmente. O Mirbase, uma base de dados de sequências e anotações de miRNAs, lista mais de 2000 miRNas humanos conhecidos (BARTEL, 2004; KOZOMARA; GRIFFITHS-JONES, 2011; MALIZIA; WANG, 2011).

A nomenclatura dos miRNAs é definida pelo Mirna Registry e miRbase Registry o qual determinam nos nomes dos miRNAs com 3 ou 4 letras como prefixo para identificar a espécie, por exemplo, hsa-miR-30 para humanos e mmu-miR30 para camundongos (GRIFFITHS-JONES, 2004; GRIFFITHS-JONES et al., 2006). As sequências maduras desses miRNAs são chamadas de 'miR", já seus precursores em formato hairpin são denominados "mir" (GRIFFITHS-JONES et al., 2006)

A partir do DNA, no núcleo, há a formação de transcritos primários de miRNAs (pri-miRNA) por meio da enzima RNA polimerase II. Ainda no núcleo celular, a enzima Drosha cliva o pri-miRNA em pré-miRNA (miRNA maduro) que migra para o citoplasma onde sofre ação da Dicer (ribonuclease do tipo III). A Dicer origina um miRNA de fita dupla que contém tanto a fita do miRNA maduro quanto a fita anti-senso (GRISHOK et al., 2001; LEE et al., 2003). O miRNA maduro pode ser acoplado à RISC (complexo de silenciamento induzido por RNA) formado principalmente por proteínas da família Argonauta. Uma das fitas duplas, miRNA maduro, permanece acoplada ao complexo RISC e atua nos seus miRNAs alvos onde conduzirá para a repressão do mRNA ou degradação do mRNA, a outra fita de miRNA, fita anti-senso, segue para sua degradação (AMBROS, 2004; BARTEL, 2004; SCHWARZ et al., 2003).

Além disso, os pré-miRs podem ser incorporados em pequenas vesículas (exossomos) ou podem ser liberados da célula por microvesículas encontradas na circulação na forma livre, ou associados com lipoproteínas de alta densidade (HDL) ou, ainda, com proteínas de ligação ao miRNA como a Argonauta-2 (Ago2) conforme exemplificado na Figura 4 (CREEMERS; TIJSEN; PINTO, 2012).

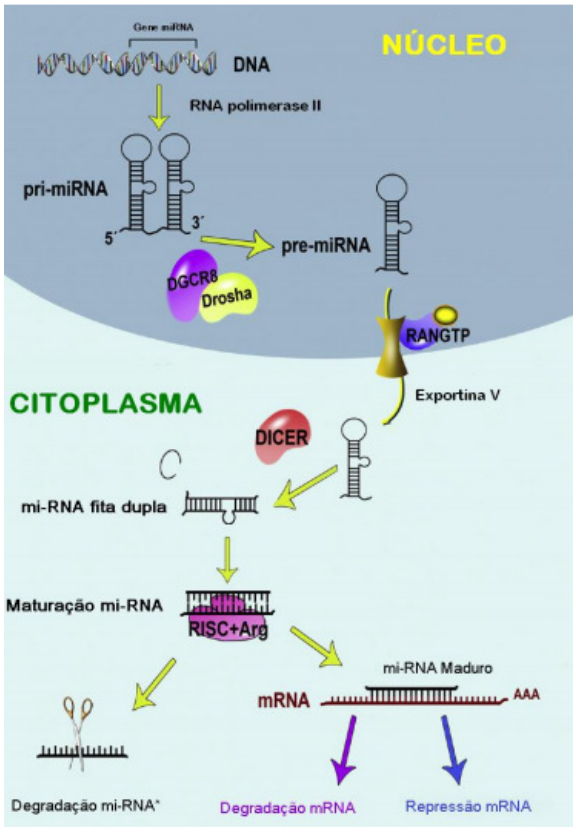

Figura 4. Formação do miRNA adaptado de Orenes- Piñero e colaboradores (ORENES-PIÑERO et al., 2013)

Os miRNAs têm papel importante tanto no processo de HC fisiológica quanto na patológica. Na última década, foi demonstrado o perfil de expressão de miRNAs em condições experimentais ou clínicas na HC identificando os miRNAs com seus genes alvos hipertróficos. Estudos identificaram miRNAs anti-hipertróficos (miRNA-1, -133, -26, -9, -98, -29, -378 e -145) e miRNAs pro-hipertróficos (miRNA-143, -103, -130a, -146a, -21, -210, -221, -222, -27a / b, -199a / b, -208, -195, -499, -34a / b / c, -497, -23a e -15a / b) no coração (DA COSTA MARTINS; DE WINDT, 2012; ELIA et al., 2009; NEVES et al., 2014). A regulação anormal do miRNA tem se mostrado envolvida nas doenças cardíacas, sugerindo que miRNAs podem afetar a estrutura e a função do coração (DA COSTA MARTINS; DE WINDT, 2012; NEVES et al., 2014; OOI;

BERNARDO; MCMULLEN, 2014). O estresse cardíaco, por exemplo, promove ativação de genes fetais e crescimento de cardiomiócitos sendo acompanhado pela expressão de miRNAs como o miR-21 *in vitro* (DIVAKARAN; MANN, 2008). No modelo patológico desenvolvido por bandeamento aórtico em animais *knockout* para miR-208 mostrou que esses animais não apresentaram hipertrofia cardíaca comparado com os selvagens provavelmente devido a falta de atividade do miR, o que mostra a influência desse miR na hipertrofia (VAN ROOIJ et al., 2007). Dentre os miRNAs citados anteriormente, quatro são reconhecidos como específicos para o coração: miRNA-1, -133a / b, -208a / be -499, chamados miomiRs. Sayed e colaboradores mostraram que o miRNA-1 cardíaco é regulado negativamente em corações hipertróficos quando HC induzida por constrição aórtica transversa (TAC) e está envolvido no crescimento muscular pós-mitótico (SAYED et al., 2007). Carè e colaboradores também observou uma redução do miR-133 em corações hipertróficos induzidos por TAC (CARÈ et al., 2007). Outros pesquisadores sugeriram que os miRNAs são importantes reguladores do crescimento cardíaco pós-natal. No caso o miR-195, um membro da família miR-15, se relaciona com a parada mitótica pós-natal de cardiomiócitos diminuindo a regulação dos genes do ciclo celular (PORRELLO et al., 2011). A indução de expressão do miR-208a visando a sinalização do hormônio tireoidiano regula negativamente a expressão do β-MHC por meio da proteína 1 associada ao receptor de hormônio da tireoide (THRAP1) (CALLIS et al., 2009; VAN ROOIJ et al., 2007). Entretanto, a expressão do miR-27a é capaz de silenciar o receptor β1 do hormônio tireoidiano, o que regula negativamente a expressão do gene β-MHC (NISHI et al., 2011).

Os miRNAs são essenciais em diferentes processos celulares envolvidos na regulação de fenótipos cardiovasculares, como crescimento de cardiomiócitos, remodelamento e vascularização (DA COSTA MARTINS; DE WINDT, 2012; FISH et al., 2008; SMALL; OLSON, 2011). Os miRNAs também têm sido descritos como participantes das adaptações benéficas promovidas pelo treinamento físico, principalmente a hipertrofia cardíaca fisiológica conforme resume a tabela 1.

Autores mostraram que a expressão dos miomiRs miR-1 e miR-133 também parecem ser regulados negativamente em modelos animais adultos de hipertrofia cardíaca fisiológica (CARÈ et al., 2007). Também observamos que miRNA-1 e -133a / b são igualmente regulados negativamente na hipertrofia cardíaca excêntrica induzida por dois diferentes protocolos de treinamento em natação quando comparados com o grupo sedentário (SOCI et al., 2011). Independentemente do exercício (corrida ou natação) e treinamento de volume (moderado e alto), os perfis de expressão desses miomiRs foram semelhantes entre os estudos (CARÈ et al., 2007; SOCI et al., 2011). Curiosamente, como descrito acima, esses miRNAs também foram reduzidos em hipertrofia cardíaca patológica (CARÈ et al., 2007).

Tabela 1. Expressão de miRNAs na hipertrofia cardíaca fisiológica e patológica

Referência	Expressão de miRNAs	Genes alvos	Fenótipo
(CARÈ et al., 2007; ELIA et al., 2009)	↓ miRNA-1 ↓ miRNA-133a	*IGD-1, IGF-1R, RHOA, CDC42, NELFA*	HC Fisiológica
(FERNANDES; SOCI; OLIVEIRA, 2011; SOCI et al., 2011)	↓ miRNA-1 ↓ miRNA-133a/b	*RHOA, CDC42, NELFA*	HC Fisiológica
(MELO et al., 2014; SOCI et al., 2011)	↑ miRNA-29a/c	*COLAGENO I/III*	HC Fisiológica
(FERNANDES; SOCI; OLIVEIRA, 2013)	↓ miRNA-208a	*PURβ*	HC Fisiológica
(FERNANDES et al., 2011)	↑ miRNA-27a/b ↓ miRNA-143	*ECA* *ECA2*	HC Fisiológica
(MARTINELLI et al., 2014)	↓ miRNA-27a ↓ miRNA-143 ↓ miRNA-26b ↑ miRNA-150	*GATA4* *IGF-1, PI3K* *GS3K-β, C-MYB*	HC Fisiológica
(MA et al., 2013)	↑ miRNA-21, -144 ↑ miRNA-145 ↓ miRNA-124	*PTEN* *TSC2* *PI3K (p110α)*	HC Fisiológica
(RAMASAMY et al., 2015)	↑ miRNA-30e ↑ miRNA-133b ↑ miRNA-208a ↓ miRNA-99b ↓ miRNA-100	*BCL-2* *IGF-1R, AKT, MTOR* *IGF-1R, AKT, MTOR*	HC Fisiológica
(LIU et al., 2015)	↑ miRNA-222	*P27, HIPK1, HMBOX1*	HC Fisiológica
(IKEDA et al., 2009; ZHAO et al., 2007)	↓ miRNA-1	*HAND2, HDAC4, CAM, MEF2A, GATA4*	Anti-Hipertrofia
(CHENG et al., 2007; ROY et al., 2009; SAYED et al., 2007; TATSUGUCHI et al., 2007; THUM et al., 2008; VAN ROOIJ et al., 2006)	↑ miRNA-21	Desconhecido	Controverso
(LIN et al., 2009)	↑ miRNA-23a	*MURF1*	Pró-hipertrofia
(BROWN; DEL RE; SUSSMAN, 2006; CARE et al., 2007; KE et al., 2004)	↓ miRNA-133	*CDC42, RHOA, NELFA/WHSC2*	Anti-Hipertrofia

(VAN ROOIJ et al., 2006)	↑ miRNA-195	Desconhecido	Pró-hipertrofia
(VAN ROOIJ et al., 2007)	↑ miRNA-208	THRAP1	Pró-hipertrofia

A fim de explicar as diferenças entre HC patológica e fisiológica baseada em assinaturas de miRNA, Lin e colaboradores identificaram miRNAs diferencialmente expressos em HC fisiológica usando camundongos transgênicos com atividade elevada de PI3K, em comparação com HC patológica o qual apresentou diminuição da atividade de PI3K. Portanto, o estudo demonstrou um papel potencial desses miRNAs na promoção de efeitos cardioprotetores no crescimento fisiológico (LIN; WEEKS; GAO, 2010).

Estudos têm mostrado uma série de miRNAs, baseado em assinaturas de miRNAs, em HC induzida por treinamento físico aeróbico (DA et al., 2012; FERNANDES et al., 2011; SOCI et al., 2011). Soci e colaboradores mostraram que a expressão de miRNA-29c, que tem como alvo o gene do colágeno, aumentou paralelamente à HC induzida por ambos os protocolos de treinamento de natação (moderado e alto volume de exercício) correlacionada com a diminuição da expressão de colágeno I e III e a concentração de hidroxiprolina relevantes para função do VE (SOCI et al., 2011). Assim, o miRNA-29 reduz a fibrose de colágeno no coração fisiologicamente hipertrófico. Por outro lado, baixos níveis de miRNA-29 foram previamente associados à fibrose no infarto do miocárdio (VAN ROOIJ et al., 2008). Recentemente, um estudo mostrou que o treinamento de natação restaurou os níveis cardíacos de miRNA-29a e -29c e preveniu a expressão do colágeno tipo I e III na borda e nas regiões remotas do infarto do miocárdio, sugerindo que o efeito cardíaco

do treinamento físico em ratos infartados do miocárdio pode prevenir ou minimizar os efeitos nocivos das doenças cardíacas (MELO et al., 2014).

No remodelamento fisiológico do VE, há crescimento coordenado tanto da massa muscular quanto da angiogênese (LAUGHLIN; BOWLES; DUNCKER, 2012). Dessa forma, pesquisadores têm investigado o papel do miRNA-126 na angiogênese cardíaca induzida pelo treinamento de natação (DA et al., 2012). O treinamento físico é capaz de promover um aumento na expressão de miRNA-126 e repressão de seus genes-alvo *Spred-1* e *Pi3k* (FISH et al., 2008). Além disso, estudo com ratos hipertensos submetidos a treinamento físico mostram níveis musculares reduzidos de miR-16 e miR-21 (que têm como alvo o fator de crescimento endotelial vascular - *Vegf* - e *Bcl-2*, respectivamente), mas níveis normais de miR-126 (que tem como alvo PI3K) (FERNANDES et al., 2012).

Outro estudo de nosso grupo observou a redução de miRNA-26a expressão após 7 dias e aumento na expressão de miRNA-150 após 35 dias de exercício voluntário. Os genes-alvo preditos do miRNA-26b e -150 podem estar envolvidos na HC fisiológica induzida pelo treinamento físico, uma vez que estão relacionados a vias de sobrevivência, como a via do IGF-1 / PI3K e glicogênio sintase quinase-3□, respectivamente (MARTINELLI et al., 2014). Curiosamente, Ma e colaboradores identificaram miRNAs que têm como alvo a via de sinalização PI3K/AKT/mTOR na hipertrofia cardíaca induzida por treinamento de natação. Os autores observaram que o treinamento físico aumentou a expressão do miRNA-21 e -144 cardíaco associado a uma redução no gene-alvo da fosfatase e homólogo de tensinas (regulador negativo da via PI3K / Akt / mTOR) (MA et al., 2013). Além disso, um aumento no miRNA-145 foi

acompanhado por uma diminuição no complexo de esclerose tuberosa 2 (TSC2) após o treinamento de natação (outro regulador negativo do PI3K /Via Akt / mTOR). Em contraste, o treinamento físico foi capaz de reduzir a expressão de miRNA-124 cardíaco associada a um aumento seu gene alvo PI3K (p110-□) envolvido na HC fisiológica (MA et al., 2013). Recentemente, outro estudo também realizou microarranjo de miRNA em tecido cardíaco de ratos saudáveis, após natação, o qual mostrou que os miR-30e, -133b e -208 foram significativamente aumentados, no entanto o miR-99b e -100 foram significativamente reduzidos inclusive após confirmação por PCR em tempo real. Ainda nesse estudo os genes alvo que regulam a proliferação morte celular foram evidenciados o que sugeriu o envolvimento de vias de crescimento como PI3 / Akt / mTOR, MAPK e a sinalização p53 na HC fisiologia (RAMASAMY et al., 2015). Tendo em vista o exposto, esses dados sugerem que o treinamento físico, pode promover HC fisiológica pela regulação de genes alvo específicos por miRNAs. Ainda há outros miRNAs reguladores da HC fisiológica, contudo mais estudos são necessários para demonstrar sua relação com o crescimento fisiológico do músculo cardíaco.

1.5 Análise computacional e ferramentas para síntese da literatura

Diante de um número grande e crescente de miRNAs relacionados ao processo hipertrófico, ferramentas de bioinformática têm sido utilizadas para auxiliar na identificação de miRNAs potencialmente envolvidos com maior relevância em processos de interesse. Sabe-se que os miRNAs quando ligados aos mRNA dependem de complementaridade da sequência. Então ao se ligar no mRNA, o miRNA provoca a

degradação do seu mRNA alvo quando a complementaridade é perfeita, contudo em uma complementaridade imperfeita a tradução do mRNA é inibida (BARTEL, 2004; NILSEN, 2007). A maioria dos miRNAs liga-se na região 3'-UTR do mRNA alvo de forma imperfeita, o que leva a uma repressão traducional. Essa inibição é considerada o mecanismo de regulação da expressão gênica dos miRNAs em animais. Quanto a evidência biológica gerada pela redução do miRNA traduzido é possível observar a partir da quantidade de proteína produzida devido ação do miRNA no mRNA alvo (NILSEN, 2007). Portanto, a ação dos miRNAs ocorre a partir de efeitos diretos ou indiretos na tradução de mRNAs o que implica em maior ou menor produção de proteínas (BARTEL, 2004; NILSEN, 2007).

A identificação de miRNAs e seus alvos, além da validação e um array em uma amostra de estudo possibilita o entendimento do perfil desses miRNAs, além de permitir inferir vias das quais os miRNAs podem estar envolvidos utilizando ferramentas de bioinformática. Em 2004, Lai discutiu a respeito de esforços dedicados à identificação sistemática de bioinformática de alvos de miRNA em animais (LAI, 2004). Os algoritmos realizam análises baseadas no alinhamento das sequências de miRNAs com as regiões 3'UTR do mRNA baseando-se em fatores como energia livre e/ou número de nucleotídeos pareados com algum requisito para emparelhamento com a porção 5' do miRNA (LEWIS et al., 2003). A fim de encontrar genes que podem ser regulados por miRNAs e interferir na regulação gênica há plataformas de predição de genes alvos de miRNAs disponíveis. Por meio do TargetScan é possível realizar a busca por alvos biológicos de miRNAs e calcular um score que pondera diversas características relacionadas ao alinhamento entre as sequências de mIRNAs e seus

possíveis alvos (LEWIS et al., 2003).(LEWIS et al., 2003). Adicionalmente, o miRTarBase (CHOU et al., 2018) permite a busca de miRNAs que atuam na regulação de genes de interesse, considerando interações experimentalmente validadas extraídas da literatura. Atualmente, o mirTarBase conta com mais de 400 mil interações miRNAs-alvos curadas manualmente após uma pré-seleção sistemática da literatura por meio de algoritmos de mineração de dados, sendo estas interações validadas com técnicas como western blot, northern blot, microarranjo, CLIP-seq, dentre outros. Após identificar os genes alvos dos miRNAs é realizada a análise de enriquecimento funcional com o objetivo de identificar em que vias esses genes-miRNAs estão envolvidos. O banco de dados KEGG é utilizado para identificar as vias de sinalização que estão relacionadas aos genes-miRNAs de interesse, portanto, o KEGG traz informações à respeito do papel destas moléculas na modulação das vias em que eles estão envolvidas (KANEHISA; GOTO, 2000; KANEHISA et al., 2016). A validação dos dados obtidos no microarranjo se refere à confirmação dos dados por meio de outra técnica, no caso PCR em tempo real. Contudo, para validar a ação de determinado miRNA ou gene é necessário métodos de inibição e ativação do gene ou miRNA como a técnica de uso de antagomiR. Os antagomiRs são fitas de oligonucleotídeos anti-senso que inibem o miR de interesse em ser inativado, de forma que reduz o miR endogenamente e isso permite entender sua ação na via de interesse conforme pesquisadores têm utilizado recentemente (TUTTOLOMONDO; SIMONETTA; PINTO, 2016; UCAR et al., 2012).

A importância da expressão de miRNAs em modelos de hipertrofia tem sido discutida com relação ao uso como um biomarcador. Nas doenças cardíacas há muitos estudos concentrados em entender o perfil de expressão dos miRNAs e identificá-los a

fim de, no futuro, poder utilizar essa informação como diagnóstico complementar. Mesmo assim, é importante entender como se apresenta a expressão dos miRNAs em situações saudáveis. Revisões abrangendo tanto cenário patológico quanto o fisiológico tem explorado os principais estudos publicados, contudo revisar de forma sistemática traz uma informação mais robusta para aprimorar o estudo. Não encontramos nenhum estudo que tenha usado ferramentas de síntese da leitura como revisão sistemática abordando o perfil dos miRNAs na hipertrofia cardíaca fisiológica. Fernandes e colaboradores em 2015 publica uma revisão o qual discutiu diferentes tipos de exercício e a expressão dos miRNAs nessas condições (FERNANDES et al., 2015). Nosso grupo tem interesse em refinar e compilar as informações a respeito do perfil de expressão e quais miRNAs estão presentes na hipertrofia cardíaca fisiológica segundo os estudos publicados até o momento.

Em suma, o processo de desenvolvimento da HC fisiológica envolve alterações morfológicas, hemodinâmicas, gênicas e protéicas que corroboram para a identificação e entendimento dos mecanismos que provocam esse fenótipo. Durante o processo adaptativo moléculas intracelulares e mediadores são sinalizados em resposta à demanda causada pelo estímulo aplicado, no caso abordamos o exercício por natação. Dessa forma, espera-se que, ao exercitar o modelo animal, o coração se adapte e, de forma compensatória, a sinalização molecular mantenha o equilíbrio com a finalidade de não desenvolver um processo patológico. Assim, explorar as moléculas envolvidas na homeostase durante a demanda exigida pelo exercício contribui para o conhecimento do desenvolvimento fisiológico e corrobora para comparações com modelos patológicos.

2. Justificativa

O desenvolvimento da HC fisiológica requer ativação de sinalizadores intracelulares, assim como proteínas citoplasmáticas a fim de aumentar o diâmetro dos miócitos e das fibras cardíacas. Pelo exercício crônico, como por exemplo em protocolo de natação por 4 semanas, obtém-se um fenótipo hipertrófico cardíaco homogêneo para o estudo da hipertrofia fisiológica.

Alguns marcadores envolvidos no crescimento muscular esquelético vêm sendo estudados inclusive avaliando o contexto patológico como na perda muscular. Da mesma forma, o estudo mecanismos moleculares envolvidos nas doenças cardiovasculares parece se direcionar para algumas hipóteses relacionadas a novas terapias. Entretanto, o mecanismo molecular e fisiológico envolvido no desenvolvimento da hipertrofia cardíaca induzida por exercício está sendo estudado gradualmente de forma que mais pesquisas ainda são necessárias. Marcadores hipertróficos como por exemplo a sinalização de mTOR e miostatina participam não só da indução de hipertrofia em músculo esquelético, mas também atuam em músculo cardíaco no que se refere a vias de sobrevivência celular em resposta ao exercício. Além disso, o desempenho biológico desses marcadores depende da regulação gênica que influenciará no tipo de fenótipo da hipertrofia cardíaca tornando-o fisiológico ou patológico.

Recentemente, tem sido reconhecido o papel dos miRNAs como reguladores gênicos envolvidos em diversos processos celulares, inclusive aqueles relacionados a HC fisiológica. Entretanto, o perfil de determinados miRNAs os quais podem regular ou participar da via hipertrófica em modelo fisiológico ainda não parece claro. Embora a literatura explore a ação dos miRNAs na HC fisiológica, há perfil de expressão de

miRNAs que não concordam entre autores. Além disso, alguns autores demonstram expressão de miRNAs semelhante tanto em HC fisiológica quanto patológica e a explicação para diferentes perfis mediante a mesma expressão do miRNA não é clara. Apesar de muitos estudos explorarem o contexto da HC patológica, poucos estudos relacionam os mecanismos moleculares que contribuem para a HC fisiológica. Nosso grupo tem estudado miRNAs envolvidos na HC fisiológica induzida por exercício, conforme Martinelli e colaboradores identificaram a expressão de alguns miRNAs bem como vias de sinalização relacionadas.

Portanto, revisar a literatura quanto à HC fisiológica a fim de discutir a expressão de miRNAs descrita pode induzir questionamentos quanto variabilidade e qualidade dos estudos. Também, demonstrar experimentalmente a expressão de miRNAs em modelo de HC aliando a análise gênica às ferramentas de bioinformática, acreditamos poder expandir o conhecimento tanto com relação a expressão dos miRNAs quanto a interação dos genes e vias de sinalização envolvidos em um fenótipo fisiológico.

3. Hipótese

A hipertrofia cardíaca fisiológica induzida por exercício promove expressão diferencial de miRNAs envolvidos com sobrevivência e crescimento celular.

4. Objetivo Principal

Avaliar as alterações na expressão de miRNAs associados a modelos de hipertrofia cardíaca fisiológica.

4.1. Objetivo Específico

Subprojeto 1:

- Revisar sistematicamente a literatura sobre alterações na expressão de miRNAs em modelos de hipertrofia cardíaca fisiológica;
- Avaliar a qualidade metodológica e a homogeneidade das alterações encontradas nos diferentes estudos;
- Comparar diferentes modelos de hipertrofia cardíaca fisiologica e o perfil de expressão dos miRNAs.

Subprojeto 2:

- Identificar o perfil de expressão dos miRNAs, por microarranjo, em animais submetidos a protocolo de natação;
- Validar por PCR miRNAs identificados no microarranjo;
- Identificar possíveis alvos dos miRNAs diferencialmente expressos por ferramentas de bioinformática;
- Identificar vias enriquecidas relacionadas aos genes alvo preditos e miRNAs diferencialmente expressos.

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Parte II

Artigo 1: Profile of miRNAs expression in physiological cardiac hypertrophy: a systematic review

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Running Title: miRNAs expression in physiological cardiac hypertrophy

Keywords: Swimming, miRNAs, physiological cardiac hypertrophy, exercise, running, microRNAs.

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Abstract

miRNAs are small RNAs involved which modulate *gene* expression by targeting mRNAs for post-transcriptional repression. Several essential biological processes including apoptosis, cellular differentiation, and proliferation are mainly influenced by miRNAs. There is a large discussion about participation of miRNAs in cardiovascular diseases; however, the investigation in physiological cardiac hypertrophy is unclear. This systematic review of literature aimed to summarize the miRNA expression profile associated to physiological cardiac hypertrophy. Eleven studies published up to January 2018 that evaluated miRNA profiles in physiological cardiac hypertrophy for this specific purpose were included in our analysis. Interestingly, only experimental studies were identified in this protocol. Among the 50 miRNAs analysed, 47 were differentially expressed as up and down-regulated (29 and 14 miRNAs, respectively) besides this 4 were both profiles. Curiously, 3 miRNAs were disagree and 3 miRNAs were agree between studies. Most of studies showed up-regulated miRNAs compared down-regulated miRNAs and majority of miRNAs were identified in mice specie. We conclude that despite a number of the investigation seeking to identify miRNAs expression and target genes in physiological cardiac hypertrophy, more research is needed to consolidate existing knowledge.

Keywords: Swimming, miRNAs, physiological cardiac hypertrophy, exercise, running, microRNAs.

Introduction

MicroRNAs (miRNAs or miRs) are endogenous noncoding small RNAs, which have few nucleotides (17-25) and modulate *gene* expression by targeting mRNAs for post-transcriptional repression [1,2]. MiRNAs participate in many essential biological processes including apoptosis, cellular differentiation, and proliferation [1]. These small RNAs are associated with essential pathways as related to cardiac diseases and are considered promising therapeutic targets for cardiovascular diseases [2–4]. In humans, more than 2000 miRNAs have been identified; miRNAs specifically or highly expressed in cardiac and skeletal muscle are called myomiRs including miR-1, miR-133, miR-206, and miR-208 [5–9]. MiR-208 is a myomiR expressed in cardiomyocytes and it is involved in the regulation of the myosin heavy chain (MHC) isoform during pathophysiological conditions. Additionally, the myomiR miR-206 is expressed in skeletal muscle and seems to play a role in neuromuscular interaction [1]. Recent studies showed the role of miRNAs as regulators in cardiac diseases, including hypertrophic growth in mice's heart submitted to transverse aortic constriction [10–12], in transgenic calcineurin mice [13], in mice with physiological hypertrophy [10], and human heart diseases [10,13]. Most studies evaluate miRNA expression in pathological cardiac hypertrophy, while changes in the physiological phenotype of cardiac hypertrophy needs to be better understood.

The potential role of miRNAs in settings of physiological heart growth has been addressed in various models such as to exercise, transgenic expression of PI3K or Akt, and pregnancy [14]. Several studies have identified miRNAs differentially regulated in physiological cardiac hypertrophy, and mRNA targets seem to contribute to the

physiological phenotype [15,16]. Results are quite variable across studies and it is difficult to reach a definite profile by evaluating individual results. We therefore performed this systematic review in order to summarize the miRNA expression profile associated to physiological cardiac hypertrophy.

Methods

The present systematic review was performed according to described BiSLR guide [17], and in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist [18]. The protocol is registered in the PROSPERO registry database under identification CRD42018094144.

Search strategy

Electronic databases PubMed, ScienceDirect, and EMBASE were searched for all studies reporting the miRNAs expression levels in physiological cardiac hypertrophy. The following keywords (and related terms) were used in the queries, which were individualized for each database interface: “hypertrophic, hypertrophies”; “, cardiac, ventricle”; “, right ventricle”; “left ventricle”; “cardiovascular”; “muscle”; “Mirna”; “microRNA”; “exercise”; “running”; “swimming”; “physical activity”; “myocardial”; “physiological” (**Table S1**). Search terms were defined by consensus among reviewers and were adjusted after the results obtained from the first queries. The search was restricted to English, Spanish and Portuguese, besides was completed on January 9, 2018. Reference lists of retrieved articles were also manually reviewed for any additional relevant studies.

Eligibility criteria

Original articles published from January 2000 to January 9, 2018 were considered for inclusion. Also, to be eligible for screening, studies had to fulfill the following criteria: i) have as main objective the identification of miRNAs in physiological cardiac hypertrophy; ii) include animals or human samples; iii) perform analysis of miRNAs expression. Studies that were performed exclusively *in vitro* or that did not have well described experimental design, including details on groups and samples metadata, were excluded. Finally, abstracts and review papers were not considered for this analysis, as well as original studies that did not report sufficient data regarding differentially expressed miRNAS levels, such as expression pattern (up / down-regulated) or fold change, and statistical significance.

Study selection

Three researchers from different areas (i.e., pharmacy, biomedicine and medicine) independently reviewed the articles retrieved in order to evaluate whether they were eligible. After removing duplicates, study selection was performed in four steps: i) titles screening, ii) abstracts screening, iii) diagonal reading, and iv) full-text reading [17]. For steps i) to iii), studies were selected for the next step if more than half of the reviewers approved the inclusion. In step iv), each reviewer had to independently evaluate the articles according to a score system and a set of five predefined questions: a) “does the article is about physiological cardiac hypertrophy?”; b) “has the article generated or used microRNA gene expression data from human or animal samples?”; c) “is the methodology of the article based on analysis of gene expression of cardiac tissue/blood

or uses public data?"; d) "does the article clearly identifies the experimental design, groups and types/number of samples?"; e) "does the article properly reports any list of potential expressed microRNAs and respective fold-change, p-value, or differential pattern (up / down-regulated) in the main text or supplementary material?"For each question, scores were given according to the following criteria: (0) if the article does not attend requirements of the question; (1) if it attends the question's requirements only partially; and (2) if it attends the question's requirements. Papers with an average score equal or higher than six were included in this review. This iterative process of evaluation and selection (BiSLR's "spiral model") aims to guarantee, as much as possible, the quality of the selected studies and also to minimize the risk of selection bias [17].

Data synthesis and analysis

Data were independently extracted by investigators using a standardized form. Two investigators perform analyses and when there were any disagreements, the third investigator analyzed data and solved by consensus.

A narrative summary of the results of the selected studies was produced and structured according to specific data fields, including: a) the article identification, such as author, year of publication, and country; b) characteristics of the study, such as study design, type of hypertrophy, biological material analyzed, groups definition, number of samples per group, assay used for expression quantification (technique and manufacturer, when possible), method applied for data analysis, and type of validation (if available); c) information about differential expression patterns of miRNAs, such as up / down-regulated or fold change, and (adjusted) p-value, when available. For each

study, reported genes and miRNAs were extracted from tables, figures, text, or supplements. Finally, a narrative synthesis was conducted, focusing on identifying patterns expression regarding reported miRNAs selected studies.

Results

Literature search and studies characteristics

A total of 3751 references were collected from the databases (790 from PubMed, 2122 from Embase, and 839 from ScienceDirect). Besides, we added 2 additional studies collected from other sources, including articles found after checking in other reviews. Also, the studies were retrieved from queries performed in the Gene Expression Omnibus (GEO) and the ArrayExpress Archive of Functional Genomics Data (ArrayExpress) databases. Duplicates were removed, resulting in 2923 studies.

After the screening phase, 2923 studies were considered for title evaluation. After title screening, 681 abstracts were included for the third step. Thereafter, 46 articles were submitted to the diagonal reading phase and 19 full texts were assessed for the fourth step. Finally, studies were assessed for eligibility, among which 8 were excluded for having an average score lower than six and 2 were removed due to either inappropriate research design to hypothesize about miRNAs expression in physiological cardiac hypertrophy or lack of original or even hypertrophy data. Other sources identified 2 studies, thus a total of 11 studies were included in this systematic review (Fig. 1).

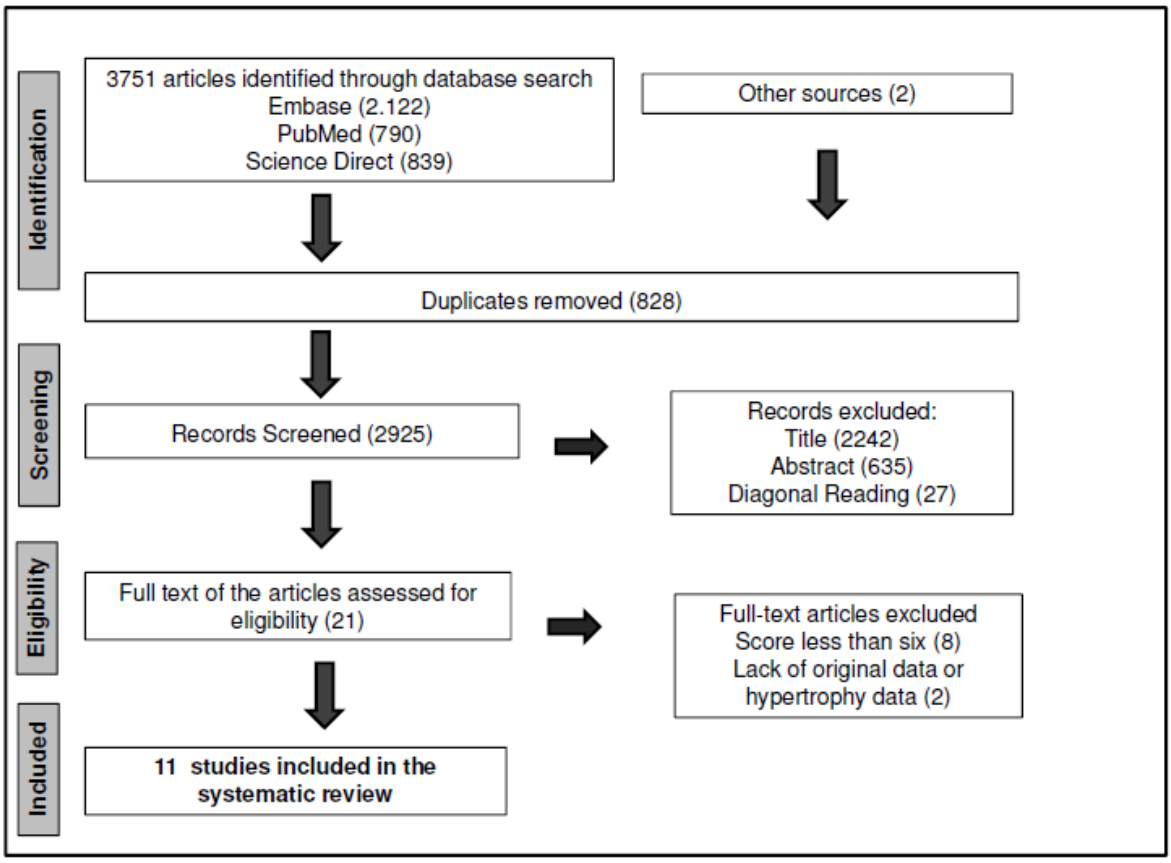


Figure 1: PRISMA flow diagram summarizing the steps of studies selection.

The main characteristics of the selected studies are provided in Table 1. From 11 studies, 54.5% are from Brazil, 9.1% are from USA, Australia, China, India, and Italy. The years of publication among the selected studies were between 2011 and 2017. The clinical articles were excluded because results did not present data regarding cardiac hypertrophy. Thus, only experimental studies were included. The protocols used to induce cardiac hypertrophy were swimming (58%), chronic training (16.7%), voluntary running (16.7%), and resisted exercise (8.3%). The sessions duration ranged from 60 min (once daily during 8 or 10 weeks) in some studies, others from 90 min (twice daily during 4 or 8 weeks), and a resisted exercise study was applied for 8 weeks. All of them

the targets analysed were miRNA and gene expression. The majority of selected studies which presented the most hypertrophy were induced by swimming. Among swimming protocols, twice daily/90min presented cardiac hypertrophy in the range of 27% to 33%; daily/60min with 5% caudal body weight resulted in the highest percentage of cardiac hypertrophy, reaching 39% of increase in heart weight.

Table 1. Characteristics of studies included in the systematic review.

Reference	Country	Exercise Training	Sample	Protocol duration	Hypertrophy degree	Target Analysed
Soci et al. 2011	Brazil	Chronic Swimming	Cardiac Tissue	T1: 60min once daily for 10wks; T2: 60min once daily for 8wks and twice daily in the 9th wk, three times daily in the 10th wk	T1: 13% T2: 27%	miRNA Gene
Fernandes et al. 2011	Brazil	Chronic Swimming	Cardiac Tissue	T1: 60min once daily for 10wks; T2: 60min once daily for 8wks and twice daily in the 9th wk, three times daily in the 10th wk	T1: 12% T2: 28%	miRNA Gene
Da Silva et al.2012	Brazil	Chronic Swimming	Cardiac Tissue	T1: 60min once daily for 10wks with 5% caudal body weight; T2: 60min once daily for 8wks with 5% caudal body weight and twice daily in the 9th wk, three times daily in the 10th wk	T1: 17% T2: 30%	miRNA Gene
Ma et al. 2013	China	Chronic Swimming	Cardiac Tissue	Once daily/60min, 5% body overload for eight wks.	39%	miRNA Gene
Martinelli el al. 2014	Brazil	Voluntary Running	Cardiac Tissue	Voluntary exercise at 7 and 35 days	Trained 7: 7% Trained 35: 11%	miRNA Gene
Melo et al. 2014	Brazil	Resisted Exercise	Cardiac Tissue	4 × 12 repetitions for eight wks.	22.2%	miRNA Gene
Ramasamy et al. 2015	India	Chronic Swimming	Cardiac Tissue	Twice daily/90min for eight wks	33%	miRNA Gene
Liu et al. 2015	USA	Chronic Swimming	Cardiac Tissue	Swimming twice daily/90min for 4 wks	29%	miRNA
Liu et al. 2015	USA	Voluntary Running	Cardiac Tissue	Voluntary running for three wks	20%,	miRNA
Soci et al. 2016	Brazil	Chronic Swimming	Cardiac Tissue	T1: 60min once daily for 10wk; T2: 60min once daily for 8wks and twice daily in the 9th wk, three times daily in the 10th wk	T1: LV 10% T2: LV 20%	miRNA Gene
Asif et al. 2017	Australia	Chronic Running	Cardiac Tissue	Once daily/60min for four wks	Juvenile: 10.3% Adolesc.: 13.5% Adult: 8.41%	miRNA
Zaglia et al. 2017	Italy	Chronic Running	Cardiac Tissue	Once 60min for eight wks	22%	miRNA Gene

miRNAs expression profile in cardiac hypertrophy

As shown in Table 2, the majority of selected studies of physiological cardiac hypertrophy used rat models (72.7%). Table 2 and Figure 2 resume mains findings on individual miRNAs. Fifty miRNAs were evaluated in at least one study. MiR-1, miR-21, miR-27a, miR-133b, miR-143, miR-208b, miR-222, and miR-499 were analyzed by more than one author and results regarding their expression were quite variable. MiR-1 expression was unchanged in chronic training in rats, however in chronic swimming was down-regulated in both rat and mice. MiR-21 was increased during chronic swimming/rat and unchanged in voluntary running/mice. Interestingly, miR-27a was different in the same specie once Liu et al[19] shown in chronic swimming and voluntary running (up-regulated), however Martinelli et al[20] shown down-regulated in mice. Ramasamy [21] found increased miR-133b in chronic swimming while Soci[15] described as down-regulated in rat and there was no difference in Asif[22] study during chronic training. miR-143 was down-regulated in chronic swimming and voluntary running besides rat and mice.

The miR-208b presented a different pattern in chronic training (down-regulated) as compared to chronic swimming (up-regulated) in two studies using rat models [21,23] . Curiously, Soci et al found miR-208b down-regulated in chronic swimming in rats [23]. The swimming and voluntary running protocols which Liu et al developed have shown results in the same line, an increased expression related to miR-210, miR-222, miR-320, miR-331-3p, miR-342-3p, and miR-484 [19]. Only miR-339-3p in Liu study was down-regulated in voluntary running and up-regulated in chronic swimming in mice [19]. It

was observed no changes in miR-499 in both mice and rat samples in different exercise protocols [20,23].

Table 2: Description of miRNAs with reported expression in physiological cardiac hypertrophy (all included studies).

miRNAs	References	Specie	Exercise Training	Change of Expression
miR-1	Asif et al. 2017	Rat	Chronic Running	Unchanged
	Soci et al. 2011	Rat	Chronic Swimming	Down
	Zaglia et al. 2017	Mice	Chronic Running	Down
miR-15a	Liu et al. 2015	Mice	Voluntary Running	UP
miR-16	Liu et al. 2015	Mice	Voluntary Running	UP
miR-19b	Ramasamy et al. 2015	Rat	Chronic Swimming	Unchanged
miR-21	Ma et al. 2013	Rat	Chronic Swimming	Up
	Martinelli el al. 2014	Mice	Voluntary Running	Unchanged
miR-22	Ramasamy et al. 2015	Rat	Chronic Swimming	Unchanged
miR-26b	Martinelli el al. 2014	Mice	Voluntary Running	Down
miR-27a	Liu et al. 2015	Mice	Chronic Swimming	UP
	Liu et al. 2015	Mice	Voluntary Running	UP
	Martinelli el al. 2014	Mice	Voluntary Running	Down
	Fernandes et al. 2011	Rat	Chronic Swimming	Up
miR-27b	Fernandes et al. 2011	Rat	Chronic Swimming	UP
miR-29a	Soci et al. 2011	Rat	Chronic Swimming	Up
miR-29b	Soci et al. 2011	Rat	Chronic Swimming	Unchanged
miR-29c	Soci et al. 2011	Rat	Chronic Swimming	Up
miR-30e	Ramasamy et al. 2015	Rat	Chronic Swimming	Up
miR-99b	Ramasamy et al. 2015	Rat	Chronic Swimming	Down
miR-100	Ramasamy et al. 2015	Rat	Chronic Swimming	Down
miR-124	Ma et al. 2013	Rat	Chronic Swimming	Down
miR-126	Da Silva et al.2012	Rat	Chronic Swimming	UP
miR-133a	Soci et al. 2011	Rat	Chronic Swimming	Down
miR-133b	Asif et al. 2017	Rat	Chronic Running	Unchanged
	Ramasamy et al. 2015	Rat	Chronic Swimming	Up
	Soci et al. 2011	Rat	Chronic Swimming	Down
miR-139	Liu et al. 2015	Mice	Chronic Swimming	UP

Table 2. Continued

miR-143	Fernandes et al. 2011	Rat	Chronic Swimming	Down
	Martinelli el al. 2014	Mice	Voluntary Running	Down
miR-144	Ma et al. 2013	Rat	Chronic Swimming	Up
miR-145	Ma et al. 2013	Rat	Chronic Swimming	Up
miR-150	Martinelli el al. 2014	Mice	Voluntary Running	Up
miR-155	Liu et al. 2015	Mice	Chronic Swimming	UP
miR-181a	Ramasamy et al. 2015	Rat	Chronic Swimming	Unchanged
miR-186	Liu et al. 2015	Mice	Chronic Swimming	UP
	Liu et al. 2015	Mice	Voluntary Running	UP
miR-191	Liu et al. 2015	Mice	Voluntary Running	UP
miR-191a	Ramasamy et al. 2015	Rat	Chronic Swimming	Unchanged
miR-193	Liu et al. 2015	Mice	Chronic Swimming	Down
miR-195	Martinelli el al. 2014	Mice	Voluntary Running	Unchanged
miR-208a	Soci et al. 2016	Rat	Chronic Swimming	Down
miR-208b	Asif et al. 2017	Rat	Chronic Running	Down
	Ramasamy et al. 2015	Rat	Chronic Swimming	Up
	Soci et al. 2016	Rat	Chronic Swimming	Down
miR-210	Liu et al. 2015	Mice	Chronic Swimming	UP
	Liu et al. 2015	Mice	Voluntary Running	UP
miR-214	Melo et al. 2014	Rat	Resisted exercise	Down
miR-222	Asif et al. 2017	Rat	Chronic Running	Unchanged
	Liu et al. 2015	Mice	Chronic Swimming	UP
	Liu et al. 2015	Mice	Voluntary Running	UP
miR-320	Liu et al. 2015	Mice	Chronic Swimming	UP
	Liu et al. 2015	Mice	Voluntary Running	UP
miR-328	Martinelli el al. 2014	Mice	Voluntary Running	Unchanged
miR-331-3p	Liu et al. 2015	Mice	Chronic Swimming	UP
	Liu et al. 2015	Mice	Voluntary Running	UP

Table 2. Continued

miR-339-3p	Liu et al. 2015	Mice	Voluntary Running	Down
	Liu et al. 2015	Mice	Chronic Swimming	UP
miR-341	Martinelli el al. 2014	Mice	Voluntary Running	Unchanged
miR-342-3p	Liu et al. 2015	Mice	Chronic Swimming	Up
	Liu et al. 2015	Mice	Voluntary Running	UP
miR-484	Liu et al. 2015	Mice	Chronic Swimming	UP
	Liu et al. 2015	Mice	Voluntary Running	UP
miR-486	Liu et al. 2015	Mice	Chronic Swimming	UP
miR-499	Martinelli el al. 2014	Mice	Voluntary Running	Unchanged
	Soci et al. 2016	Rat	Chronic Swimming	Unchanged
miR-547	Liu et al. 2015	Mice	Chronic Swimming	UP
miR-574-3p	Liu et al. 2015	Mice	Voluntary Running	UP
miR-652	Liu et al. 2015	Mice	Voluntary Running	UP
miR-680	Martinelli el al. 2014	Mice	Voluntary Running	Unchanged
miR-1224	Martinelli el al. 2014	Mice	Voluntary Running	Unchanged

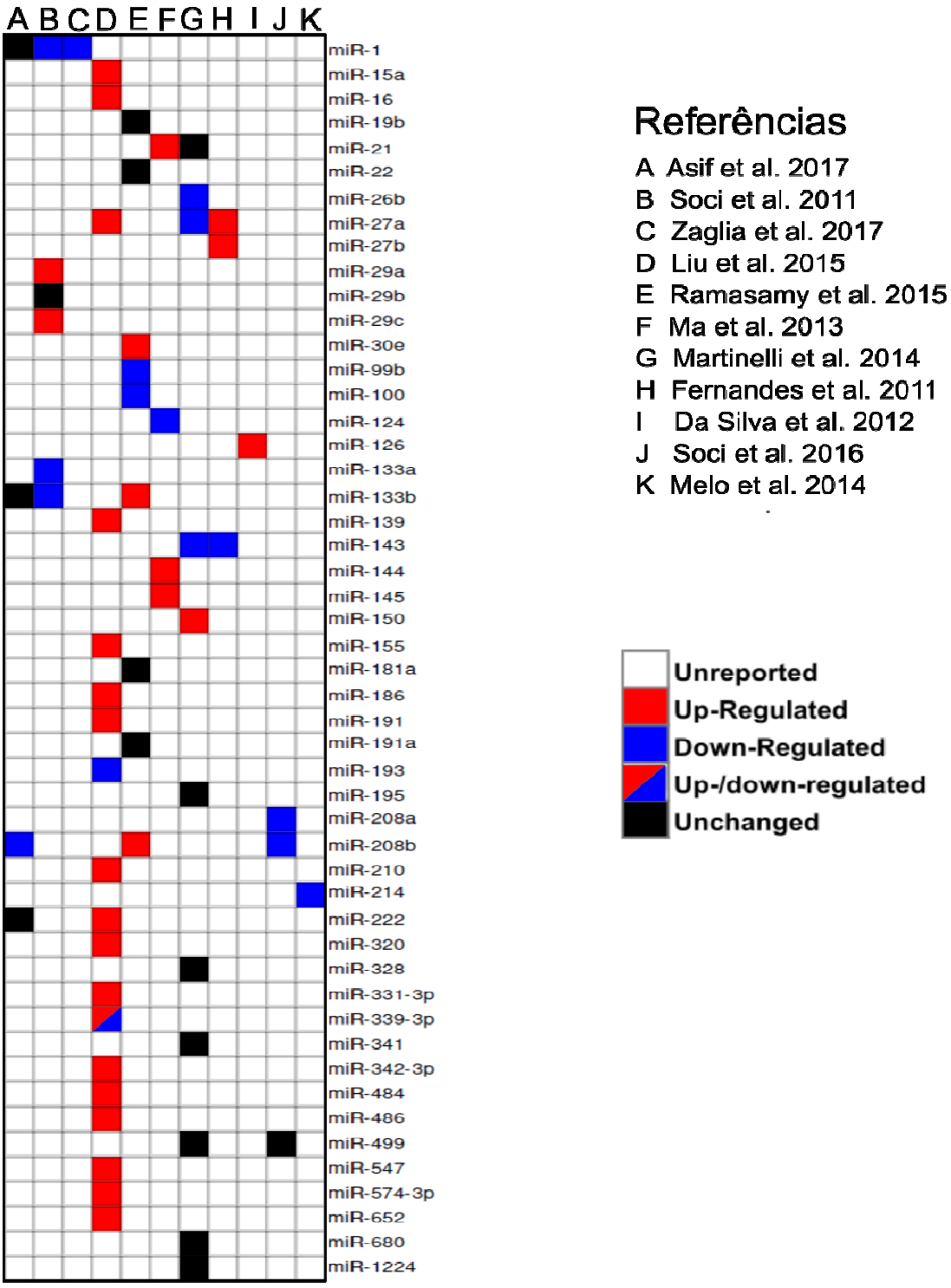
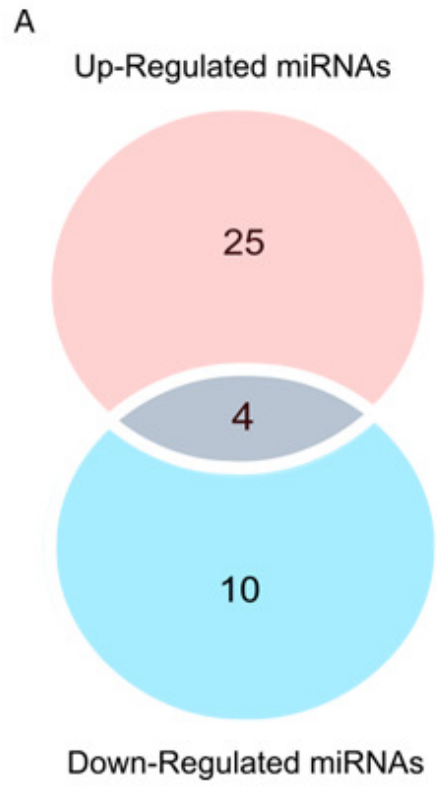


Figure 2. Graphical representation of all reported miRNAs and their changes in physiological cardiac hypertrophy.

Patterns of expression

Out of 50 miRNAs reported in the eleven included studies, 47 presented either as up or down-regulated (Figure 3a). Among these miRNAs with differential expression, 29 were up-regulated, 14 were down-regulated and 4 were up-regulated in some studies and down-regulated in others (Figure 3a). In Figure 3b we present individual data for which miRNAs were up or down-regulated. Further, Figure 3C depicts the distribution of miRNAs with up and down-regulated expression according to different species.



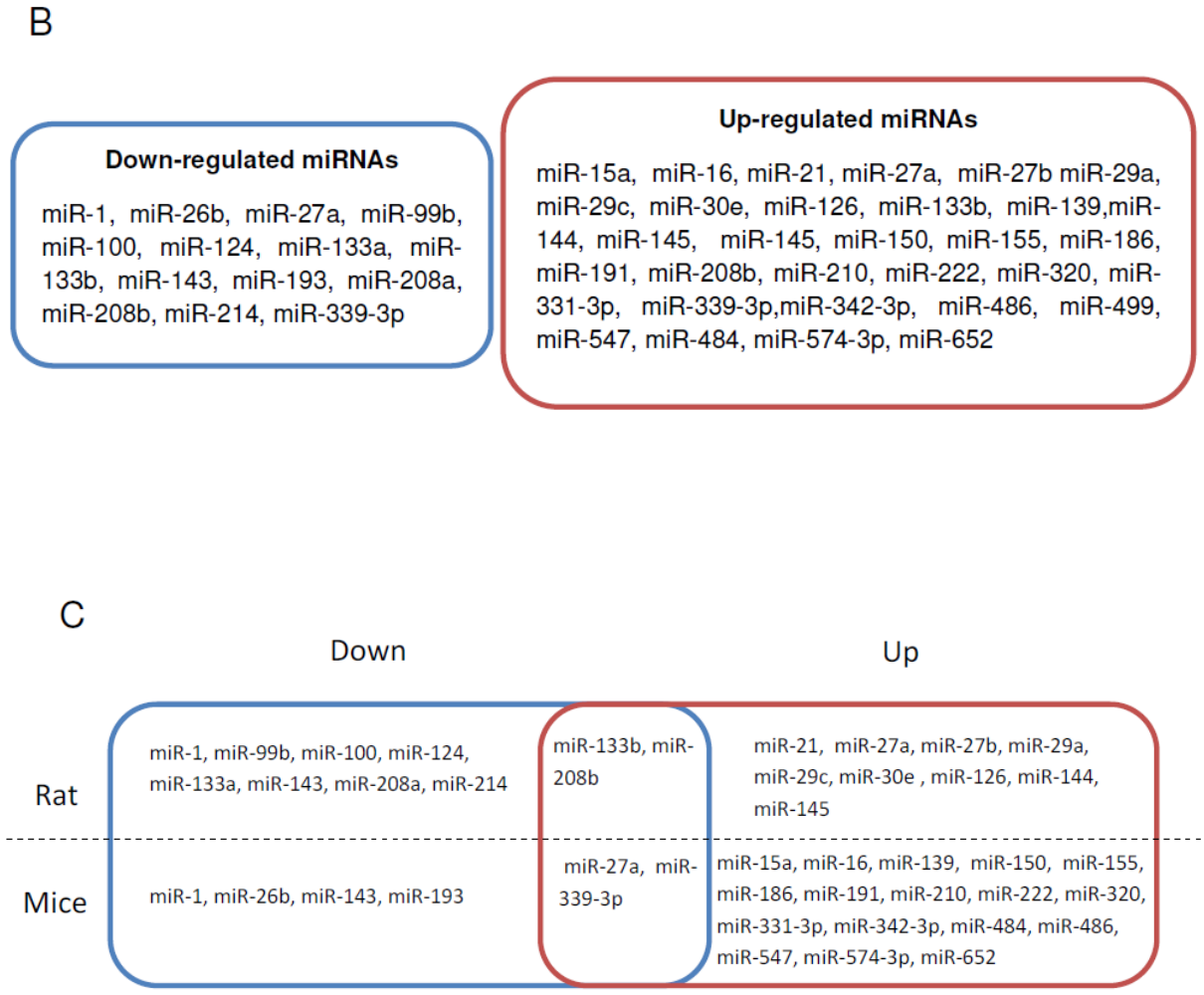


Figure 3: Comparison of differential expression patterns observed for A) miRNAs among all selected studies in a cross-organ analysis. Up-regulated is represented by red shade, while down-regulated is denoted by blue shade. B) miRNA expression profile between the studies. C) miRNA expression profile between the species.

Some miRNAs were evaluated in both rats and mice, as depicted in Table 3. Only 3 miRNAs presented a similar pattern in both rat and mouse models in more than one study: miR-143 was down-regulated in both mice and rats [20,24]. miR-1 was down-regulated in mice and either down-regulated or unchanged in rats [15,22,25]; and miR-27a

was up-regulated in rats and either down or up-regulated in mice [19,20,24]. MiR-499 was reported to be unchanged in two studies, including rat and mouse models [20,23].

Regarding miR-21 and miR-222, there was no consistente findings when comparing mouse and rat models (Table 3).

Table 3. miRNAs expression evaluated in different studies and species

miRNAs	**Mice miRNA expression**	**Rat miRNA expression**
miR-1	Down [25]	Unchanged [22] / Down [15]
miR-21	Unchanged [20]	Up [26]
miR-27a	Up / Down [19,20]	Up [24]
miR-143	Down [20]	Down [24]
miR-222	UP [19]	Unchanged [22]
miR-499	Unchanged [20]	Unchanged [23]

Evaluation of targets

Among the included studies only 9 evaluated possible target genes of miRNAs. In Figure 4 we present the number of supporting experimental evidences for target genes in all included studies. There reported changes in 25 target genes: 13 were increased and 12 were decreased. Further, supplementary Table 2 depicts miRNAs related to these genes. Fernandes et al presented miR-27a and -27b up-regulated whereas corresponding gene (*ACE*) were down-regulated. In the same study, miR-143 is down-regulated and *ACE2* target is up-regulated. Most authors validated microarray and miRNA targets by qPCR or western blotting (Supplementary Table 3).

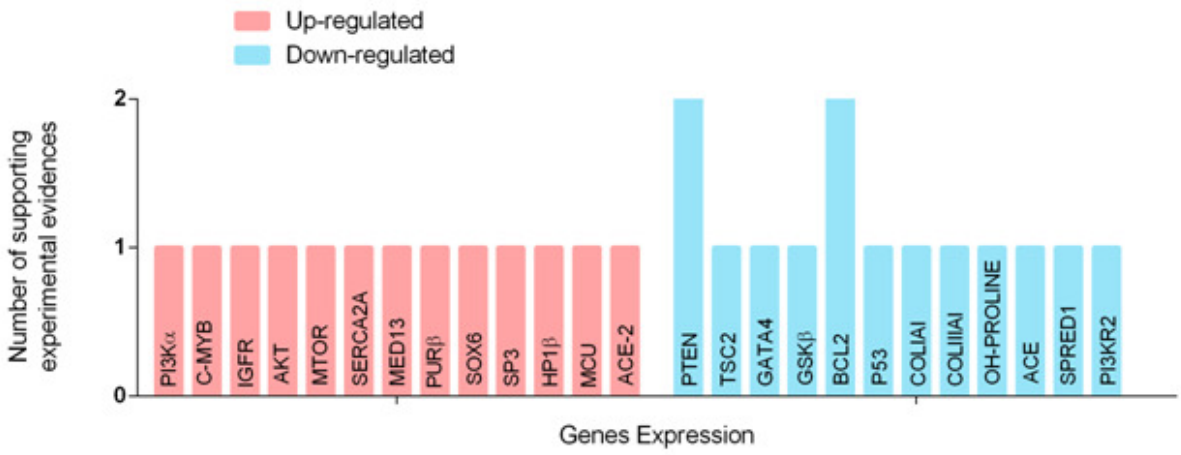


Figure 4. Target genes with reported changes in expression in physiological cardiac hypertrophy.

Discussion

In this systematic review, data from eleven studies evaluating miRNA expression in physiological cardiac hypertrophy is presented. Among 50 reported miRNAs data in more than one study was observed for only 8 miRNAs, and data in both mice and rats was reported for only six miRNAs. Furthermore, for most of the miRNAs, there were conflicting results. Only two miRNAshad results that were consistent in different studies and in both mice and rats: miR-143, which was down-regulated in both mice and rats; and miR-499 which was unchanged in both species.

Vascular Smooth Muscle Cells (VSMCs) are specialized cells that regulate the lumenal diameter of small arteries/arterioles called resistance vessels, thereby contributing to the regulation of blood pressure and are controlled by angiotensin II (ANGII) [27]. Some studies argue about a role assumed by miRNAs as effectors acting in a context-dependent, combinatorial manner [28,29]. Thus, a study reported the

miR143/145 gene cluster as a major regulator of the phenotype of VSMCs, besides it governs the levels of molecules involved in the synthetic and contractile state of Smooth Muscle Cells (SMCs) and directly affect contractility of VSMCs [30]. Moreover, the inhibition of *ACE* (angiotensin converting enzyme) has been studied to induce atherosclerotic lesion regression [31].

Therefore, Fernandes et al noticed correlation of miRNAs with *ANG*-related genes and possible implications of miRNAs regulating *RAS* genes in physiological cardiac hypertrophy has not been reported previously [24]. In this study, miR-143 was down-regulated only in protocol T2 where cardiac hypertrophy increased 28% in chronic swimming for 90 min/10 weeks in female rats [24]. The researchers discussed that miR-143 has been named as a regulator of *ACE2* gene as similarly demonstrated by the previous studies [24,30]. Thus, Fernandes identifies that aerobic exercise training exerts an effect on the expression of miRNAs and thereby might regulate their specific target genes. Martinelli et al showed identical expression for miR-143 despite different species, such as mice. However, the down-regulation expression was identified only in the group which runs for 7 days [20]. In this study, the author suggested that the duration, intensity and willingness to perform exercise considering wheel-running vs forced swimming can influence cardiac hypertrophy degree. The less intensity, due to wheel-running, impacts differently in intracellular pathways which regulate cell growth and apoptosis. According to studies by Martinelli, there are potential miR targets which play relevant role in pathways related to cell survival and hypertrophy development [20]. This study reinforced the ErbB pathway in which several numbers of targets and overlaps were presented [20]. Other investigation demonstrated that cardiac-restricted over-

expression of ErbB2 in transgenic mice led to the development of striking concentric cardiac hypertrophy [32]. Previous studies reported that cardiac ErbB2 overexpression which was able to activate protective signaling pathways, including phosphoinositide 3-kinase (PI3K)/AKT, besides conducting to an antiapoptotic shift in the heart. In this ligne, studies proposed that the PI3K/AKT pathway is directly involved in the development of physiological, but not pathological, cardiac hypertrophy [32,33]. Also, Martinelli identified the insulin signaling in physiological profile [20]. Authors have discussed Insulin-related pathways which were associated to participate in the physiologic cardiac hypertrophy by increase of insulin-like growth factor 1 (IGF-1) and insulin-like growth factor 1 receptor (IGF1R), via the PI3K (p110 alpha) pathway [34,35]. Martinelli found a down-regulated expression after 7 training days in mice and suggested that the previously established regulatory pathways controlling pathological hypertrophy are not deregulated in physiologic cardiac growth. However, more studies are necessary to validate the targets of miRNAs and understand that their functions indicate the role of miRNAs in cardiac adaptations.

MiR-499 was unchanged in two studies which evaluated mice and rats. Martinelli and other authors noticed that the miR-499 decreased in microarray analysis which did not confirm by qPCR technique. Alterations in miR-499 has been associated with cardiac diseases as reported in previous studies [36,37]. Despite miR-499 being a myomir up-regulated in pathological conditions, the exercise training was not able to inhibit this miRNA as discussed by Martinelli [20] and Soci, though exercise changes others myomirs as miR-208a and miR-208b [23]. There is a relation between miR-208 with myosin heavy chain and miR-499. Myosin heavy chain type α (α-MHC) is predominant in adult heart [38]

and encodes the miR-208a which are responsible to develop myofiber diversification, stress-responsiveness, and thyroid hormone sensitivity of the heart. In the mouse heart, miR-208a is required for up-regulation of b-MHC and miR-208b in response to stress and hypothyroidism. MiR-208a has been regulating a fetal gene myosin heavy chain 7 type b (*MHY7B* gene) and its intronic miRNA, miR-499; which are highly expressed in the adult heart in the absence of stress. Nonetheless, the axis regulation b-MHC/miR-208a commented before is different to b-MHC/miR-208b once this last axis requires stress or hypothyroidism expression. In this line, miR-208a expression plays a role during stress due to genes repression in the heart. The miR-208a controls *MYH7b*/miR-499 expression which results in 50% reduction in miR-208a$^{+/-}$ mice and is extinguished in hearts of miR-208a$^{-/-}$ mice. Forced expression of miR-499 in miR-208a$^{-/-}$ hearts restores expression of b-MHC and represses ectopic expression of fast muscle genes, consistent with the conclusion that miR-499 functions downstream of miR-208a. Also, transgenic expression of miR-499 in miR-208a$^{-/-}$ mice reactivates the expression of *MYH7b* indicative of a positive autoregulatory loop [39]. Noteworthy, it seems that miR-499 expression plays a role and depends on fetal genes expression against a pathological stimulus. Soci presented in this study a decreasing in miR-208a and miR-208b and proposed that the *α-MHC/β-MHC* expression ratio observed after exercise training occurred due to miRNA decreasing and increased expression of *Med13*, *Purβ*, and *Sox6*, repressors of *β-MHC* [23]. Although Soci and Martinelli did not evaluate fetal genes, several studies have reported the absence of this gene expression in physiological phenotype [40]. Therefore, we can suggest that miR-499 unchanged in both

studies is maybe related to absence of the fetal genes in physiological cardiac hypertrophy.

MiR-1 is a myomiR expressed in cardiomyocytes which plays a significant role in cardiac remodeling and hypertrophy function [41,42]. Soci and Zaglia found similar results such as miR-1 as down-regulated when applied chronic training by swimming or running in rat or mice [15,25]. Soci validated miR-1 expression of the microarray by qPCR while Zaglia identified this miRNA by qPCR where both confirmed reduced levels. Additionally, Zaglia et al identified *MCU* gene as a target of miR-1 while Soci only described miR-1 expression without evaluating the target gene [15,25]. In exercise training, miR-1 was down-regulated while *MCU* gene was down-regulated. Similar behavior of the miR-1/MCU axis in these conditions (exercise vs. pressure-overloaded) was discussed around the possible common upstream regulator of miR-1/MCU signaling pathway. To support this hypothesis, studies have been suggested that miR-1 depend on the β-AR/Akt/FOXO pathway [25,43,44]. Even though Asif et al evaluated miR-1 in the same species as Soci, this study was unable to find a similar result, maybe we can explain this due to different genders between studies.

MiR-222 expression was different in two studies, according to Asif et al there is no difference in moderate intensity endurance training during four weeks in rat model [22]. In healthy young athletes after both acute and chronic exercise suggesting relevance of the miR-222 in humans [45] . Liu examined changes in circulating miR-222 heart failure patients after cardiopulmonary exercise testing [19] similar to exercise found in young athletes [45]. Also, in wheel-running during 3 weeks and swimming training for four weeks (90 min/twice daily) in mice, there was an increase in miR-222 [19]. Further, in

cardiomyocyte, size in vitro which induced a physiological pattern of myosin heavy chain isoform expression (relative ratio of Myh6/Myh7 increased) [19]. Thus, miR-222 is an important miRNA in physiological and pathological cardiac hypertrophy even though the function in the heart is unknown or their levels unchanged in other species as showed in Asif's study. Based on clinical implications of miR-222, Liu suggested this miRNA as the interesting biomarker or functional mediator.

MiR-27a was reported for three studies as up-regulated in rats or mice, except one study in mice which was shown as down-regulated [19,20,24]. Fernandes et al reported about the correlation of miRNAs with angiotensin related genes, including miR-27a [24]. Thus aerobic exercise training exerts an effect on the expression of miRNAs and thereby might regulate their specific target genes. Additionally, Liu presented miR-27a up-regulated during swimming or running by mice identified in microarray and validated in real-time, similar to Fernandes with rats. However, Martinelli showed this miRNA down-regulated during 35 days of voluntary running in mice identified by microarray and confirmed by real-time only in a group which trained for 7 days. In this study it seems that miR-27a decreased only when cardiac hypertrophy was developing and seems that this change does not persist when hypertrophy is established. Given the above, there are inconsistencies regarding the profile of this miRNA in physiological cardiac hypertrophy.

In physiological phenotype, we found two studies that presented miR-21 with different levels. Martinelli reported this miRNA as unchanged before voluntary training in mice whereas Ma et al showed an increase in rats. The role of MiR-21 in cardiovascular scenery has been described by several studies [47,48]. In cardiomyocyte hypertrophy the

positive and negative roles of miR-21 has been reported [12,51]. Thus, Ma et al reported in exercise training a relation between physiological adaptations with miR-21 up-regulated and *PTEN* expression gene decreased. In this line, miR-21 seems to present the same profile in both phenotypes, whether physiological or pathological. Meantime, there is no consistency on miR-21 profile in physiological cardiac hypertrophy.

Conclusion

In conclusion, in this systematic review only few studies were identified evaluating the expression of miRNAs in physiological cardiac hypertrophy induced by exercise. Among reported miRNAs, most had been shown to have changes in expression that were not consistent across studies, either when comparing studies in the same species, as well as comparing different species. Amongst miRNAs with more consistent findings, miR-143 as down-regulated in both mice and reats, and miR-499 was unchanged in both species. More studies should be performed, preferentially comparing species and validating findings, in order to improve the knowledge in this field.

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Supplementary Tables

Table S1. Specific queries performed in the three databases assessed for this SLR.

Database	Search term
Embase	(('hypertrophy'/exp OR 'hypertrophy' AND [embase]/lim) AND ((1microrna'/exp OR 'microrna') AND [embase]/lim)
PubMed	(((“microRNAs” OR “MicroRNA” OR “miRNAs” OR “Micro RNA” OR “RNA, Micro” OR “miRNA”))) AND ((“Hypertrophy” OR “Hypertrophy, Left Ventricular” OR “Hypertrophies, Left Ventricular” OR “Left Ventricular Hypertrophies” OR “Ventricular Hypertrophies, Left” OR “Ventricular Hypertrophy, Left” OR “Ventricular Hypertrophy, Right” OR “Right Ventricular Hypertrophy” OR “Hypertrophies, Right Ventricular” OR “Right Ventricular Hypertrophies” OR “Ventricular Hypertrophies, Right” OR “Cardiac Hypertrophy” OR “Heart Hypertrophy”))
Science Direct	(((“microRNAs” OR “MicroRNA” OR “miRNAs” OR “Micro RNA” OR “RNA, Micro” OR “miRNA”))) AND ((“Hypertrophy” OR “Hypertrophy, Left Ventricular” OR “Hypertrophies, Left Ventricular” OR “Left Ventricular Hypertrophies” OR “Ventricular Hypertrophies, Left” OR “Ventricular Hypertrophy, Left” OR “Ventricular Hypertrophy, Right” OR “Right Ventricular Hypertrophy” OR “Hypertrophies, Right Ventricular” OR “Right Ventricular Hypertrophies” OR “Ventricular Hypertrophies, Right” OR “Cardiac Hypertrophy” OR “Heart Hypertrophy”))

Table S2. Summary of miRNA targets expression

ID_Interno	miRNAs	miRNA Regulation	Target Gene	mRNA Regulation
Soci et al. 2011	miR-1	Down	-	-
Soci et al. 2011	miR-133a	Down	-	-
Soci et al. 2011	miR-133b	Down	-	-
Soci et al. 2011	miR-29a	Up	-	-
Soci et al. 2011	miR-29b	Unchanged	-	-
Soci et al. 2011	miR-29c	Up	*COLIAI, COLIIIAI, OH-proline*	Down
Fernandes et al. 2011	miR-27a	Up	*ACE*	Down
Fernandes et al. 2011	miR-27b	UP	*ACE*	Down
Fernandes et al. 2011	miR-143	Down	*ACE2*	Up
Da Silva et al.2012	miR-126	UP	*Spred-1, PI3KR2*	Down
Ma et al. 2013	miR-21	Up	*PTEN*	Down
Ma et al. 2013	miR-124	Down	*PIK3α*	Up
Ma et al. 2013	miR-144	Up	*PTEN*	Down
Ma et al. 2013	miR-145	Up	*TSC2*	Down
Martinelli el al. 2014	miR-26b	Down	*IGF1R*	Unchanged
Martinelli el al. 2014	miR-27a	Down	*GATA4*	Down
Martinelli el al. 2014	miR-143	Down	-	-
Martinelli el al. 2014	miR-150	Up	*GSKβ*	Down
Martinelli el al. 2014	miR-150	Up	*C-MYB*	Up
Martinelli el al. 2014	miR-328	Unchanged	-	-
Martinelli el al. 2014	miR-341	Unchanged	-	-
Martinelli el al. 2014	miR-680	Unchanged	-	-
Martinelli el al. 2014	miR-1224	Unchanged	-	-
Martinelli el al. 2014	miR-21	Unchanged	-	-
Martinelli el al. 2014	miR-195	Unchanged	-	-
Martinelli el al. 2014	miR-499	Unchanged	-	-
Melo et al. 2014	miR-214	Down	*SERCA2a*	Up
Ramasamy et al. 2015	miR-208b	Up	*THRAP1, Myostatin*	-
Ramasamy et al. 2015	miR-19b	Unchanged	*MuRF, Atrogin-1, αCryB*	-
Ramasamy et al. 2015	miR-19b	Unchanged	*PTEN, Bcl2*	Down
Ramasamy et al. 2015	miR-133b	Up	*CyclinD, Nelf-A, RhoA, Ccd42*	-
Ramasamy et al. 2015	miR-30e	Up	*CaMKIIδ, Egfr1*	-
Ramasamy et al. 2015	miR-30e	Up	*Bcl2*	Down
Ramasamy et al. 2015	miR-99b	Down	*IGF1R, Akt, mTOR*	Up
Ramasamy et al. 2015	miR-100	Down	*IGF1R, Akt, mTOR*	Up
Ramasamy et al. 2015	miR-191a	Unchanged	*Egr1, Cd4, Casp4, SOCS4*	-
Ramasamy et al. 2015	miR-191a	Unchanged	*p53*	Down
Ramasamy et al. 2015	miR-22	Unchanged	*CDK6, Sir1, Sp1*	-
Ramasamy et al. 2015	miR-181a	Unchanged	*MAPK1, TNFα, GATA4*	-
Soci et al. 2016	miR-499	Unchanged	-	-

Soci et al. 2016	miR-208a	Down	*Med13, Purβ, Sox6, SP3, HP1β*	Up
Soci et al. 2016	miR-208b	Down	*Med13, Purβ, Sox6, SP3, HP1β*	Up
Zaglia et al.2017	miR-1	Down	*MCU*	Up

Table S3. Description of techniques and sample size in the studies.

Reference	Specie	Transcriptome profiling technique	Array samples	Array validation	miRNA validation technique	N per group in validation
Soci et al. 2011	Female Wistar rat	microarray	2	Yes	qPCR	5
Fernandes et al. 2011	Female Wistar rats	microarray	2	Yes	qPCR	7*
Da Silva et al.2012	Female Wistar rats	NO	NA	NA	qPCR	7
Ma et al. 2013	Female Wistar rat	microarray	ND	NA	qPCR	16*
Martinelli el al. 2014	Male Balb/c mice	microarray	4	Yes	qPCR	between 10-12
Melo et al. 2014	Male Wistar rat	NO	NA	NA	qPCR	7
Ramasamy et al. 2015	Wistar rats	RNA sequencing data	ND	Yes	qPCR	6
Liu et al. 2015	Male C57BL6/J mice	microarray	3	Yes: only miR-222	qPCR	4
Liu et al. 2015	Male C57BL6/J mice	microarray	3	Yes: only miR-222	qPCR	4
Soci et al. 2016	Female Wistar rat	NO	NA	NA	qPCR	between 6-7
Asif et al. 2017	Male Wistar-Kyoto rat	NO	NA	NA	qPCR	11
Zaglia et al. 2017	C57BL/6J mice	NO	NA	NA	qPCR	6

qPCR: PCR real time, NA: not applicable, ND: not described, *N each group, however in qPCR technique is not clear

Parte III

Artigo 2: miRNA expression profile and experimental validation in a model of physiological cardiac hypertrophy.

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Running Title: miRNA EXPRESSION PROFILE IN CARDIAC HYPERTROPHY

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Abstract

The cardiac muscle responds to increased workload by developing myocardial hypertrophy. MicroRNAs (miRNAs) have been implicated in myocardial hypertrophy, mostly in cardiac diseases. However, in a physiological model, research is scarce. We aimed to identify and validate changes in miRNA expression in a model of physiological cardiac hypertrophy, and to identify pathways related to differentially expressed miRNAs. Mice aged 8 weeks old were trained by swimming protocol during 7 (T7) and 28 days (T28) for cardiac hypertrophy induction. Microarray analysis were performed using the Affymetrix platform (11 samples: S28 n=5, T28 n=6). Real time PCR was performed to validate differentially expressed miRNAs. Functional enrichment was performed to evaluate the pathways involved. Exercise resulted in hypertrophy (9% in the 7 days and, 13% in 28 days), with no changes in fetal genes or increase in fibrosis, confirming the physiological phenotype. Microarray analysis identified 22 miRNAs differentially expressed in hypertrophy group: 9 were down-regulated (miR-7686-5p, -1934-3p, -7221-3p, -7047-5p, -3072-5p, -346-3p, -6937-5p, -3077-5p)and 13 were up-regulated (miR-10a-5p, -215-5p, -29c-5p, -212-5p, -21a-5p, -329-3p, -206-3p, -34b-3p, -872-3p, -434-3p, -3060-3p, -487b-3p, -1983). All changes in selected miRNAs were confirmed by RT-PCR in 28 days, and miRNA 329-30 was up-regulated both at 7 and 28 days. In target gene prediction, 14 miRNAs were related to possible hypertrophic pathways, and the pathways related to cellular growth such as insulin and MAPK were related to most identified miRNAs. In conclusion, we identified and validated several miRNAs related to physiological cardiac hypertrophy, as well as important signaling

pathways, miR-329-3p was identified for the first time as being related to physiological hypertrophyand further studies could focus in elucidating its role in this process.

Keywords: Swimming, miRNAs, physiological cardiac hypertrophy, mouse model, exercise.

Introduction

Exercise training triggers cardiac hypertrophy as a compensatory mechanism to deal with the increased energy demand during the activity [1]. Several studies revealed that exercise training promotes a physiological cardiac hypertrophy, and contributes to attenuation of the pathological cardiac hypertrophy as triggered by myocardial infarction, heart failure, and cardiomyopathy [2,3]. The cardiac hypertrophy due to chronic exercise involves molecular mechanisms with changes in genes, miRNAs and protein expression. Therefore, the physiological molecular phenotype differs from that observed in cardiac diseases. It has been shown that microRNAs (miRNAs, miRs) are regulated by exercise and they seem to contribute to physiological cardiac hypertrophy [4].

miRNAs are small non-coding RNAs, highly conserved (17-25 nucleotides) whose production is endogenous [5,6] . Additionally, their function is to regulate gene expression by decreasing gene or protein expression post-transcriptionally. MiRNAs have been identified by Mirbase list with over 2000 known human miRNAs and they have been shown to be implicated in the process of cellular senescence, inflammation and in cardiovascular disease [5,7,8]. Regarding adaptive regulation growth, some miRNAs are muscle specific such as miR-1 and miR-133, which are decreased in physiological cardiac hypertrophy [9], whereas miR-208 is considered specific to cardiac muscle and is less abundant than miR-1 [10]. Cardiovascular diseases are frequently studied and consider the miRNAs as promising therapeutic targets in this scenery. Also, the miRNAs have been known for having a key role in physiological hypertrophy by regulating cellular functions as, for example, decreasing fibrosis and apoptosis or cell growth and angiogenesis [11]. Different miRNAs and their potential pathways were

identified due to exercise stimulation, however, in physiological cardiac hypertrophy, the expression profile is still unclear. We hypothesized that the miRNA levels may be changed in a model of physiological cardiac hypertrophy influencing different signaling pathways which contribute to approach a physiological profile. In this study, we identify and validate miRNAs differentially expressed in physiological cardiac hypertrophy and analyze the most likely pathways expressed.

Methods

Animal Care

All protocols were approved by Animal Care and Use Committee of Hospital de Clínicas de Porto Alegre (GPPG-120250) and were performed as recommended Guidelines for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health[12,13] This manuscript was written in accordance with the Guide for the Care and Use of Laboratory Animals, the ARRIVE guidelines [12,13] . A total of 52 male BALB/c mice aged around 8 weeks (73.6 days ± 17.3), basal body weight 26.0 g (25.7g ± 1.7) were used in this study. The animals were housed in controlled-temperature conditions (22 ± 2°C), under a 12:12 light-dark cycle, and received standard chow and water ad libitum.

Animals were randomly allocated per batch and box as described in Supplementary methods. After the setting in the boxes, animals were allocated for cage to sedentary or exercise-training groups. Animals were evaluated at early (7 days) or established (28 days) follow-up, thus generating four groups: S7 (sedentary 7 days, n=12), S28 (sedentary 28 days, n=12), T7 (trained 7 days, n=13), and T28 (trained 28 days, n=15).

In order to calculate the sample size, the means and standard deviations of the molecular evaluations of the preliminary study were considered (60% of increase in miRNA levels, with a standard deviation of 40%). These data were used from a previous study performed by our group that measured miRNAs expression in physiological hypertrophy protocol during 7 and 35 days of voluntary physical exercise [14]. Therefore to obtain a power of 80% and alpha error probability of 5%, the sample size calculated was a minimum of 8 mice per group at each time (7 and 28 days). The primary experimental outcomes assessed were cardiac hypertrophy and miRNAs levels, however, miRNA levels were considered for size calculation. The secondary outcomes assessed were body weight, fibrosis, fetal genes, and signalling pathways.

Chronic Swimming Training

Swimming training protocol for mice was adapted from Evangelista et al [15] with some modifications. Briefly, training consisted of swim for 90 minutes twice daily, 5 days/week, during a total of 7 (T7 group) or 28 days (T28 group), with water temperature at 30-32°C. On the first, the mice were adapted 3 days as progressive time: 5 minutes - day 1, 10 minutes - day 2, 15 minutes -day 3. Then, on the initial day of protocol started of 20 minutes; the duration was increased by 10 min every day until day 5 and by 15 minutes in days 6 and 7 twice day. All subsequent sessions lasted 90 min- twice day. To minimize the influence of water stress, sedentary animals were placed in the pool for 5 minutes twice a week during the experimental protocol. An adapted pool was built for implementation of this protocol. Swimming apparatus for mouse physical training adapted from Evangelista et al [16]. The system is composed of a

glass tank measuring 10 mm thick, 60 cm wide, 100 cm long, and 50 cm high. The inner tank is made up of 4-mm glass divided into 9 groups with a surface area of 30x15 per lane and 35-cm depth to allow individual training. To avoid flotation of the animal during swimming, a bubble system was used, consisting of a water pipe connected to an air pump which was coupled to the lower base of the inner tank. An automated heating system maintained the temperature between 30–32°C, and a filter with 420 L/h capacity was used to keep the apparatus clean.

Assessment of Physiological Hypertrophy

After 7 and 28 days of swimming, the animals were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg). The hearts were harvested 24h after the last training session. Both atria and the right ventricle were immediately excised in order to isolate the left ventricle (LV), which was measured as the left ventricular weight/tibial length ratio (LVW/TL in mg/mm) and left ventricular weight / body weight ratio (LVW/BW in mg/g). Tibias were collected and radiographed by a trained technician. The radiographs were scanned and tibial length was performed by Image Pro-Plus (version 4.1.0.0 Media Cybernetics, L.P.).

The LV was sliced and were stored in a RNA later solution (Qiagen, Inc., Austin, USA), after 24h the RNA later solution was removed and the sample was immediately frozen in liquid nitrogen. Other slice was fixed in 10% buffered formalin, and then paraffin-embedded.

Fibrosis analysis. Paraffin-embedded heart sections (4-5 µm thick) were prepared and stained with Masson's Trichrome to estimate cardiac fibrosis. Four

random microscopic fields from light microscopy (200x magnification) were photographed using Q Capture Software v.2.81.0 (Q Imaging Co., CA). Fibrosis was quantified using the color deconvolution plug-in of Image J 1.45s software (n=5-8 per group). Fibrosis intensity was calculated according to the formula: f=255–I, where f is final blue intensity and I is the mean blue intensity varying from 0 (dark blue, highest expression) to 255 (white, no expression). All histological analysis were performed by one blinded and independent investigator.

miRNAs and mRNA Extraction

A Total RNA and miRNAs were extracted from 30 mg of LV using the MiRNeasy Mini kit (Qiagen, Inc., Austin, USA) according to the manufacturer's instructions. Big RNAs and the enriched portion with miRNAs were stored at -80 ºC for subsequent molecular analyses. The concentration of RNA and miRNAs samples was assessed using the 260/280 nm ratio (NanoDrop™ 1000 Spectrophotometer – Thermo Fisher Scientific, USA).

mRNA and miRNAs by quantitative real-time PCR (qRT-PCR)

Before perform microarray, fetal genes were analysed to support choice of samples and prove physiological cardiac hypertrophy in this protocol. Therefore, fetal genes *Nppa*, *Myh6* and *Myh7* were evaluated using TaqMan Gene Expression Assay probes and measured by real-time PCR (Applied Biosystems, Inc., USA). Assay numbers (MM01255747_g1, MM00440359_m1, MM01319006_g1) and the data was normalized for glyceraldehyde 3- phosphate dehydrogenase gene (*Gapdh*).

After selected miRNAs that were significantly difference in microarray data were chosen for validation. First-strand cDNA samples were synthesized from miRNAs using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Inc., USA), according to the manufacturer's instructions. RT-qPCR and miRqRT-PCR reactions were run in duplicate with QuantStudio™ 3 Real-time PCR System, using TaqMan miRNA expression assays (Applied Biosystems Inc., USA), following the manufacturer's instructions. The $2^{-\Delta Ct}$ was used to calculate relative gene expression according described previously [17].

miRNAs Microarray

A sub-sample of 11 animals (S28, n=5; T28, n=6) were subjected to microarray miRNA analysis due to the objective of screening the miRNA profile. We choose those samples which presented high cardiac hypertrophy (established) based on cardiac assessments. In the supplementary table 1, we show data related morphological characteristics considered on the samples chosen to GeneChip array. Samples (150 ng of total miRNA) were subjected to analysis on the GeneChip Scanner 3000 7G Affymetrix platform. Hybridization, washing, and staining were performed using GeneChip® Hybridization, Wash, and Stain Kit (GeneChip® miRNA 4.0 Array and Flashtag™ Bundle, Affymetrix). Scanning was performed using GeneChip Scanner 3000 7G Affymetrix. We also included the GeneChip® Hybridization Control Kit for controls and normalization purposes. All the procedures were executed according to the manufacturer's instructions.

Array data analysis

The CEL files containing miRNAs raw expression data were pre-processed and analyzed with R/Bioconductor packages to obtain normalized expression profiles. The RMA algorithm from the oligo package [18] was applied to perform background correction, quantile normalization, and probeset summarization. Probe annotation was downloaded from Affymetrix support material website. Since miRNA 4.0 is a multi-species array, all probes unrelated to mouse were removed prior to further analyses. Two-group comparison of normalized expression data, expressed on log-scale, was performed with Welch's t-test to investigate differentially expressed (DE) miRNAs related to physiological cardiac hypertrophy. Probes presenting a statistically significant (p-value < 0.05) two-fold change ($logFC > 1$ or $logFC < -1$) in expression levels between groups were considered as DE among groups. To account for multiple testing, p-values were corrected using the Benjamini and Hochberg False Discovery Rate (FDR) method and the corresponding FDR values are reported in results tables. Hierarchical clustering was performed using complete linkage and Euclidean distance as a measure of similarity for the differentially expressed miRNAs.

miRNAs Target Prediction and Functional Enrichment Analyses

To investigate the potential role of differentially expressed miRNAs, we searched for putative targets using a combination of experimentally validated and computationally predicted interactions derived from public databases and bioinformatics tools. miRTarbase release 6.1 [19] was queried for validated miRNA targets restricting for interactions classified as functional, including those with weak experimental evidence.

Predicted targets were collected from three computational prediction tools, namely starBase v2.0 [20], TargetScan (release 6.2) [21], and Diana Micro T v4.0 [22]. To reduce false positive rates, the following filters were applied: (i) for starBase v2.0, we required target sites to be predicted by at least three out of the five softwares in addition to the experimental support from CLIP-Seq data; (ii) for TargetScan (release 6.2), only predictions with total context score less than -0.2 and context score percentile higher than 50 were kept; (iii) for Diana MicroT v4.0, predictions were filtered using a score cutoff of 0.7. In addition, unlike TargetScan and Diana MicroT, starBase brings together experimental validation and computational prediction information to provide a list of potential targets.

All miRNAs and mRNAs identifiers were mapped to miRBase v21 and Human Gene Nomenclature Committee [23,24] standards, respectively, and the combination of unique validated and predicted miRNA-target interactions was used for further analyses.

Furthermore, we performed functional enrichment analysis of retrieved miRNA targets using pathways annotation from the KEGG Pathway Database [25,26] and the clusterProfiler package in R/Bioconductor environment [27]. Statistical significance for KEGG pathways enrichment was estimated with a hypergeometric test and adjusted to account for multiple hypotheses testing using the FDR procedure. Pathways with a p-value < 0.05 were highlighted as potentially enriched for DE miRNAs in physiological cardiac hypertrophy. Interactions between differentially expressed miRNAs and putative target genes were visualized using Cytoscape v3.7.0 and pathview R/Bioconductor package.

Statistical Analysis

The normality of all data were analysed by Kolmogorov-Smirnov test. Therefore, normal data are presented as mean ± SEM or mean ± SD and parametric tests was applied using two-sample Student *t*-test. However, abnormal data are presented as median and interquartile range and non-parametric tests were performed by Mann-Whitney test. Comparisons were considered between two independent groups (S7 *vs* T7 or S28 *vs* T28) and differences were considered significant when $p< 0.05$. Data were analyzed using GraphPad Prism Version 6.0.

Results

Exercise training developed physiological cardiac hypertrophy

All animals allocated to the training program tolerated and adapted well to the swimming protocol, with no adverse events or death in the protocol groups. Trained animals were lighter than the sedentary group, which can be explained by the caloric loss related to the daily swimming protocol. Physiological cardiac hypertrophy was observed by the hypertrophy index (left ventricular weight corrected by tibia length - LVW/TL ratio) in which trained groups presented an increase of about 9% at early stage (T7: 6.0 ± 0.3 mg/cm; S7: 5.5 ± 0.1 mg/mm; $p= 0.17$), and a 13% increase at established cardiac hypertrophy (T28: 6.1 ± 0.1 mg/mm; S28: 5.3 ± 0.1 mg/mm; $p= 0.0001$) according to Table 1. When we considered cardiac hypertrophy by left ventricular weight corrected by body weight (LVW/BW ratio) the trained groups presented an increase of about 9% and 26% according to Table 1 in cardiac

hypertrophy. Animals trained for 7 days did not change the body weight. For this reason we only discuss LVW/TL ratio. The swimming protocol was performed to induce physiological cardiac hypertrophy, therefore to confirm the physiological cardiac development profile we evaluated fibrosis in the left ventricle. Thus, there was no presence of fibrosis in the groups (Table 1).

Table 1. Cardiac Hypertrophy Assessments. Values are presented as mean ± SD and comparisons were performed using Student *t*-test, *p<0.005. N per group = 5-15 mice each group (S7 *vs*. T7 group, S28 *vs*. T28 group).

Table 1 Cardiac hypertrophy Assessments

Groups	BW (g)	LV (mg)	LVW/BW (mg/g)	LVW/TL (mg/mm)	Fibrosis (A.U.)
S7	27.1 ± 1.96	87.7 ± 2.70	3.2 ± 0.16	5.5 ± 0.35	157.9 ± 18.01
T7	26.7 ± 1.22	96.2 ± 2.58*	3.6 ± 0.32*	6.0 ± 0.65	157.3 ± 10.80
S28	28.6 ± 2.36	88.3 ± 2.71	3.0 ± 0.27	5.3 ± 0.55	139.1 ± 10.60
T28	26.0 ± 1.90*	100.1 ± 1.57*	3.8 ± 0.27*	6.0 ± 0.34*	141.2 ± 16.05

Each data are mean ± SEM (Student *t*-test). N per group = 5-15 mice each group. * P<0.005, S7 vs.T7 group, S28 vs. T28 group. BW body weight, LV left ventricular, LVW left ventricular weight, TL tibial length.

Additionally, we performed fetal genes expression to confirm the physiological profile in group T28 which was chosen for microarray analysis. In this way, fetal genes *Myh7, Myh6 and Nppa* did not change in T28 (Fig.1).

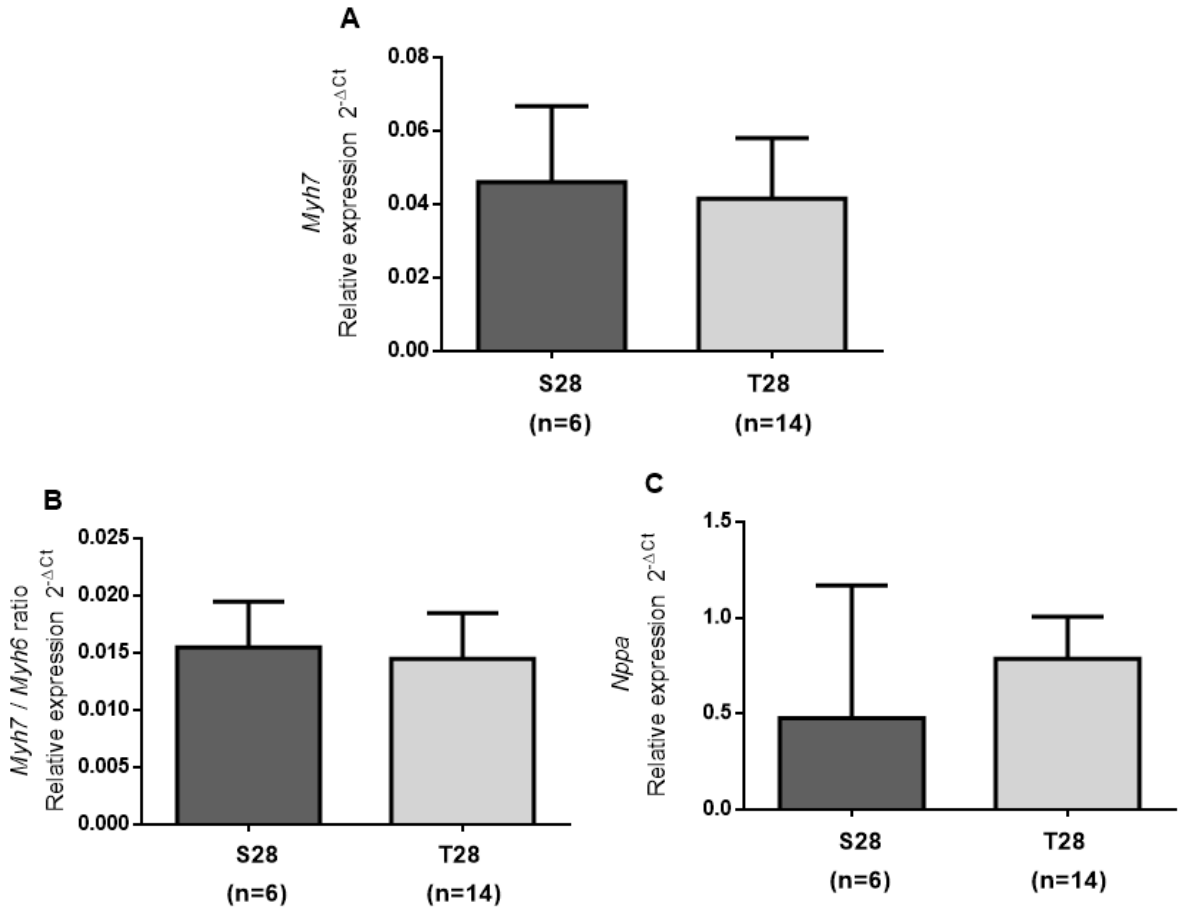


Figure 1. Fetal genes expression in sedentary and trained mice at 28 days. S28: sedentary at day 28; T28: trained at day 28. Data were expressed as median and interquartile range (Mann-Whitney).

Identification of differentially expressed miRNAs associated with exercise training

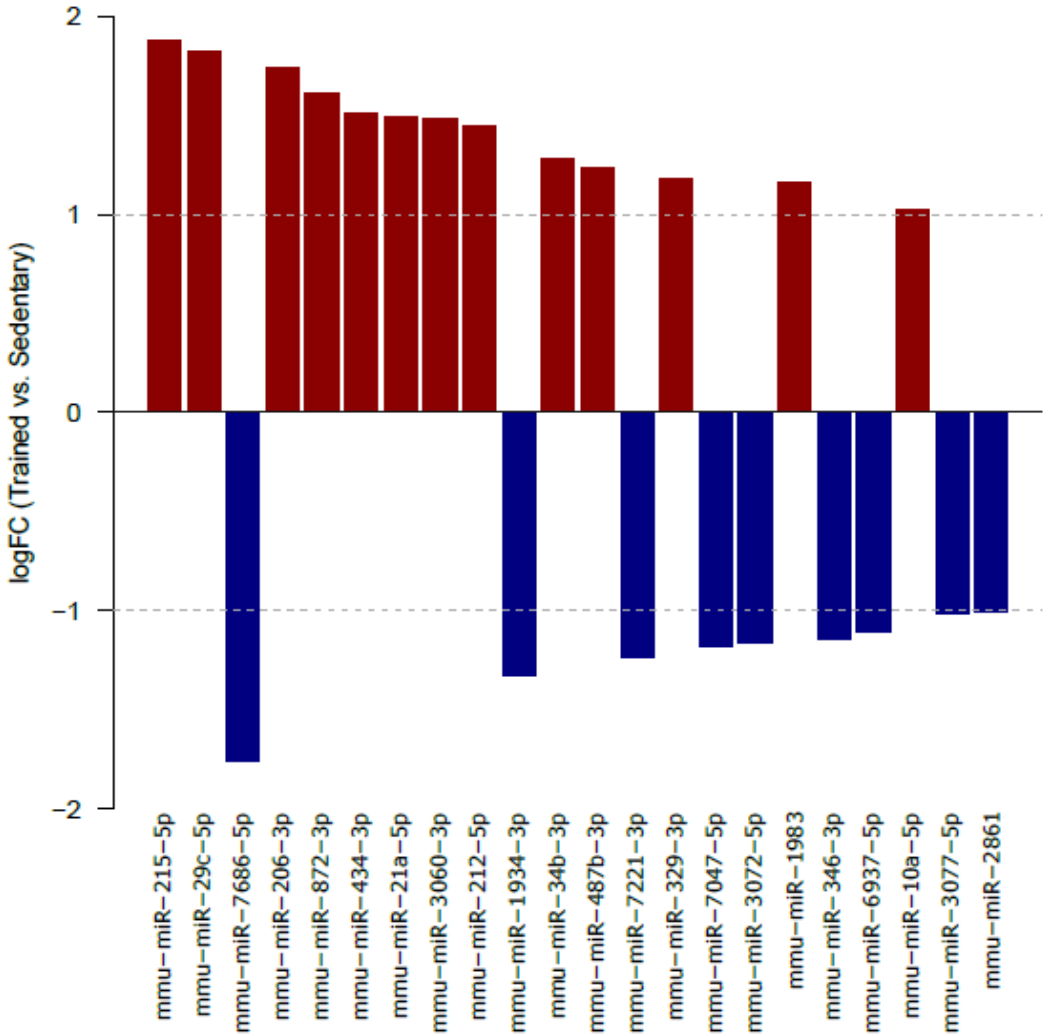
As explained above, miRNAs participate in the biological process including cardiovascular disease and exercise training [8,9,11]. To identify miRNAs expressed in cardiac tissue, microarray analysis was performed to screen miRNAs expression in sedentary and trained groups and hypothesize about differentially expressed miRNAs in physiological cardiac hypertrophy. A total of 3163 mouse-related probes from miRNA 4.0 array, comprising 1255 miRNAs precursors (pre-miRNAs) and 1908 mature miRNAs, were evaluated and compared among trained and sedentary groups (n=5-6 per group). Statistics of differential expression analysis are given in Supplementary table 2. The volcano plot representation (Supplementary Figure 1) summarizes the results, in which statistically significant up-regulated probes (i.e., p-value < 0.05 and logFC > 1) are represented as red points, whereas significant decreased (i.e., p-value < 0.05 and logFC < -1) probes are denoted as blue points.

Only a small portion of probes presented a minimum two fold change in expression levels in trained vs. sedentary samples. Among these, 22 mature miRNAs were considered to be differentially expressed among groups according to the pre-defined threshold for the statistical significance (Figure 2A). A total of 13 miRNAs were up-regulated in trained animals vs. sedentary (red bars), whereas nine miRNAs (blue bars) presented down-regulated expression associated to physiological cardiac hypertrophy.

We observed miRNAs which presented around 2 fold change as miR-206-3p, miR-29c-5p, and miR-215-5p (2.02, 1.81 and 1.87 fold change, respectively). However, the others miRNAs presented fold change between 1.02 and 1.75 (Fig.2A).

A heatmap visualization and hierarchical clustering analysis of gene expression levels considering the differentially expressed miRNAs (Figure 2B) suggested a grouping tendency among samples according to the presence of physiological cardiac hypertrophy, except for samples 23 and 51, which were improperly clustered together.

A



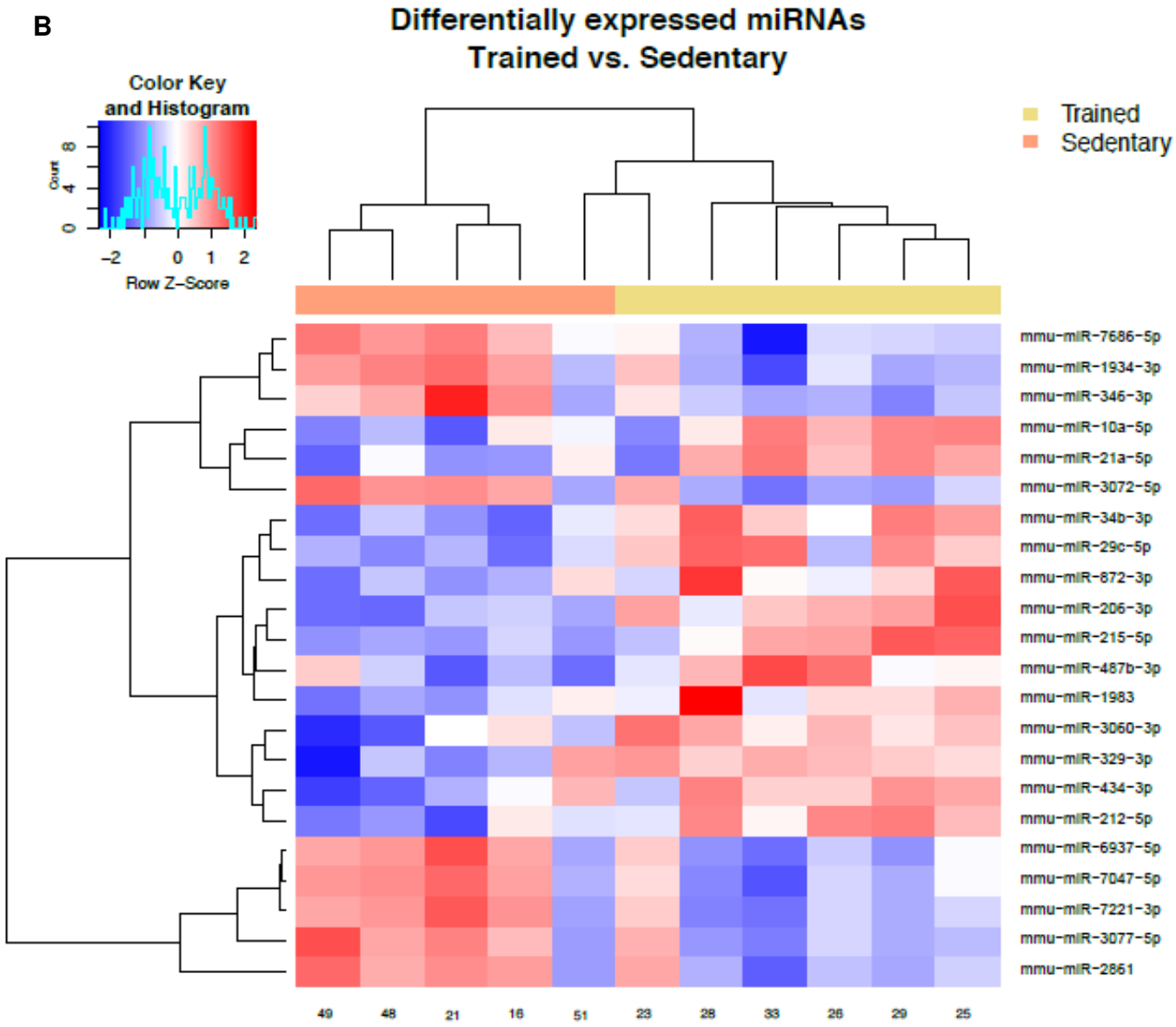


Figure 2. Microarray expression analysis. (A) Differentially expressed miRNAs identified in the microarray analysis comparing trained (T28, N = 6) and sedentary (S28, N = 5) samples, according to a p-value < 0.05 and an absolute log-fold change (logFC) higher than 1 (i.e., 2-fold change cutoff.). Up-regulated miRNAs are denoted in red, whereas down-regulated miRNAs are shown in blue. (B) Heatmap and clustering analysis of differentially expressed miRNAs showing expression patterns of differentially expressed miRNAs (p-value < 0.05 and |logFC| > 1) for trained and sedentary samples. Higher expression levels are represented in red, while lower expression values are represented in blue. Hierarchical clustering of miRNAs and samples was performed using complete linkage and Euclidean distance as a measure of similarity.

Putative targets of differentially expressed miRNAs and KEGG pathway analysis

To explore and understand the relationship of miRNAs differentially expressed with physiological cardiac hypertrophy, we analyzed their putative target genes and enriched pathways through bioinformatic approaches. For this purpose, separated analyses were performed for target genes of up/down-regulated miRNAs.

By using a compilation of experimentally validated and computationally predicted interactions from miRTarBase, starBase, TargetScan, and Diana MicroT resources, we obtained a total of 5444 miRNAs-target gene interactions regulated by 14 out of the 22 differentially expressed miRNAs. According to the adopted filtering criteria, no target genes were found for mmu-miR-29c-5p, mmu-miR-7686-5p, mmu-miR-1934-3p, mmu-miR-7221-3p, mmu-miR-7047-5p, mmu-miR-3072-5p, mmu-miR-6937-5p, and mmu-miR-3077-5p. Among the retrieved interactions, 1117 derived from experimental evidence and 4327 originated from computational prediction tools (Supplementary table 3).

The total number of targets per miRNA, as well as the number of targets according to the type of supporting evidence, are provided in Table 2. Most of the targets found were related to up-regulated miRNAs, with only two decreased miRNAs (i.e., miR-346-3p and miR-2861) comprised by this list. A total of 3854 and 930 unique target genes were found to be collectively regulated by up/down-regulated miRNAs, respectively, with 212 common targets between both sets.

Table 2. Putative targets of differentially expressed miRNAs. Number of targets retrieved through bioinformatics analysis for each miRNA using experimental and computational prediction evidences

MiRNA	Total number of targets	Targets from experimental evidences	Targets from computational prediction
mmu-miR-10a-5p	640	247	393
mmu-miR-1983	384	20	364
mmu-miR-206-3p	459	97	362
mmu-miR-212-5p	629	22	607
mmu-miR-215-5p	264	13	251
mmu-miR-21a-5p	272	61	211
mmu-miR-2861	923	10	913
mmu-miR-3060-3p	391	15	376
mmu-miR-329-3p	1020	579	441
mmu-miR-346-3p	8	---	8
mmu-miR-34b-3p	176	11	165
mmu-miR-434-3p	190	37	153
mmu-miR-487b-3p	87	5	82
mmu-miR-872-3p	1	---	1

According to our analyses, a number of genes related to cardiac hypertrophy and cellular survival were found to be regulated by the differentially expressed miRNAs. *Rictor* gene is regulated by miR-212-5p, -329-3p, -206-3p, and -215-5p, all of them with increased expression in physiological cardiac hypertrophy (Supplementary table 3). *Bcl2l11* is an experimentally validated target of miR-10a-5p and -329-3p, whereas *Mtor* is a predicted target of miR-212-5p. Additionally, computational prediction suggested miR-34b-3p as a regulator of *Hsp90aa1* gene, on the other hand *Bcl2* and *Pik3r1* as target genes of miR-10a-5p and miR-206-3p, respectively (Supplementary table 3). We found *Vegfa*, *Atg10,* and *Pik3r1* as potential targets of miR-329-3p, whereas *Pten* was observed to be a predicted target of miR-3060-3p and miR-10a-5p and it was an experimentally validated target of miR-21a-5p. Finally, *Mapk1* was retrieved as a predicted target gene of miR-215-5p (Supplementary table 3).

Functional enrichment analysis of putative target genes using annotations from KEGG Pathway database was performed to identify molecular mechanisms under modulation of miRNAs regulated by exercise. A total of 104 pathways were enriched (p-value < 0.05) for target genes of down-regulated miRNAs (Supplementary table 4). Among the significant pathways, 57 had strong enrichment (FDR < 0.05), including FoxO, MAPK, Insulin, mTOR, PI3K-Akt, Autophagy - animal, AMPK, EGFR tyrosine kinase inhibitor resistance, and Apoptosis signaling pathways. On the other hand, 12 pathways were found to be associated with targets of down-regulated miRNAs, none of which presented a strong enrichment (i.e., FDR < 0.05). We note that this difference in the number and the statistical significance of enriched pathways for up and down-regulated miRNAs may be due to the lower number of target genes for the latter, which

affects the statistical power of the test. Ras, Regulation of actin cytoskeleton, Insulin, Insulin resistance, and Hypertrophic cardiomyopathy signaling pathways were over-represented among targets of down-regulated miRNAs (Supplementary table 4).

The relation of the top enriched pathways ($p < 0.05$) for targets of up and down-regulated miRNAs, and their corresponding statistics (p-value and gene ratio), are summarized in Figure 3. For better visualization purposes, KEGG pathways under "Human diseases" category and directly related to cancer, were omitted from this figure given their irrelevance for the current study. We can observe that signaling pathways by Insulin-PI3K-Akt, MAPK, and Ras have the participation of the largest proportion of targets for up-regulated miRNAs in T28 vs S28 groups. Ras signaling pathway is also enriched for target genes of down-regulated miRNAs, although with lower statistical significance (Fig.3).

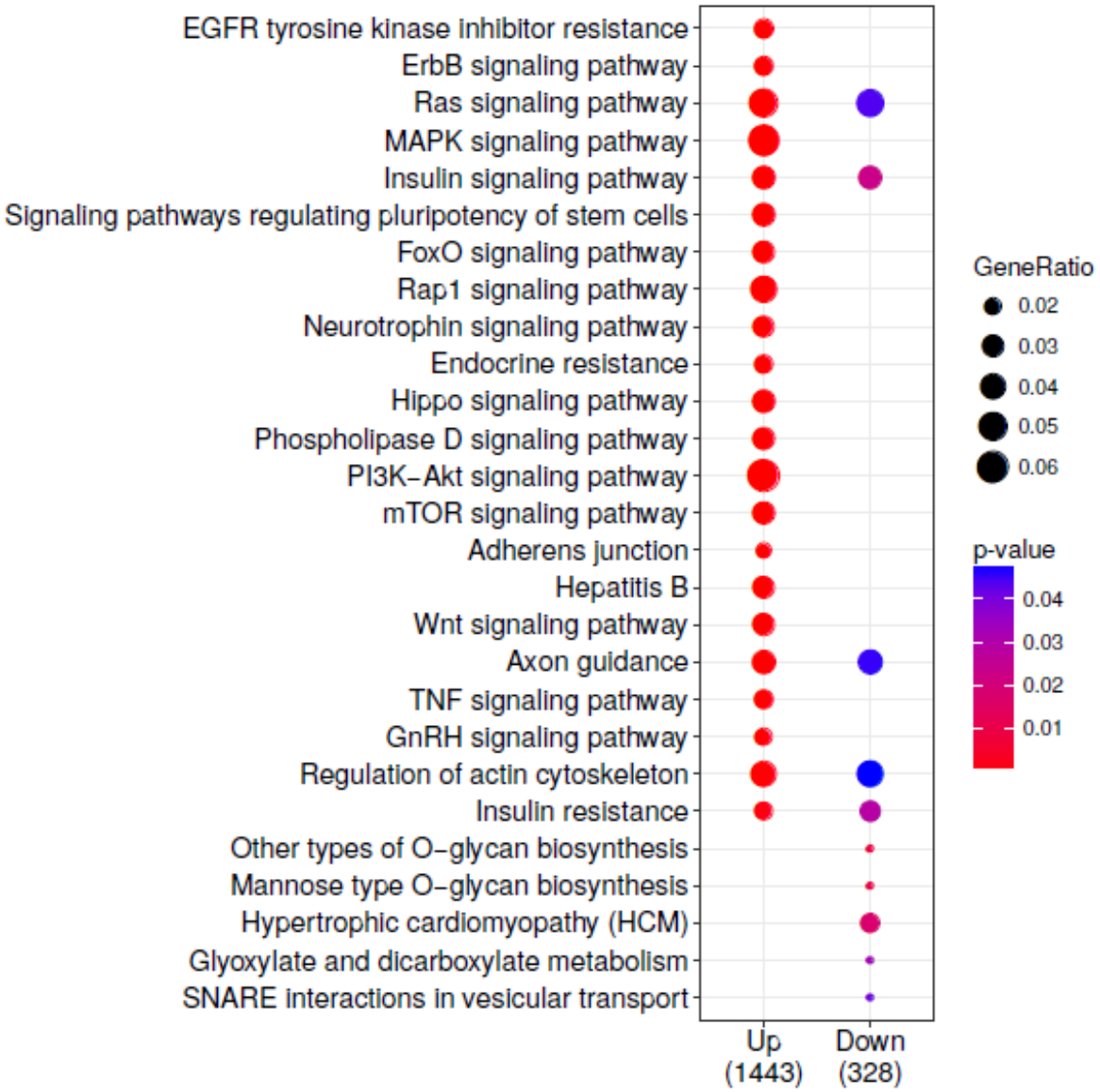


Figure 3. Top KEGG pathways enriched (p < 0.05) for target genes of differentially expressed miRNAs. Among the retrieved putative targets of up/down-regulated miRNAs expression, 1443 and 328 genes, respectively, were annotated in the KEGG pathways database. Gene ratio is specified by circles' size and reflects the proportion of targets participating in a given over-represented pathway, while circles' colors denote the corresponding p-value of the enrichment test.

Validation of microarray-based differentially expressed miRNAs

A subset of miRNAs differentially expressed in the array was chosen for further validation based on their differential expression patterns, as well as previous findings from the related literature. Therefore, the miRNAs validated were chosen according to the current literature which did not present a validation in cardiac tissue. Also, we considered to chose of the miRNAs involved with previously pathways described related to cardiac hypertrophy. Thus, miR-206-3p, miR-34b-3p, miR-29c-5p, miR-215-5p, miR-212-5p, miR-21a-5p, miR-329-3p, and miR-10a-5p were chosen to be validate.

Validation of array-based differentially expressed miRNAs results are depicted as relative expression $2^{-\Delta Ct}$ [17]. The assays analyzed were: mmu-miR-10a-5p (ID 000387), mmu-miR-215-5p (ID 001200), mmu-miR-29c-5p (ID 001818), mmu-miR-212-5p (ID 461768), mmu-miR-21a-5p (ID 000397), mmu-miR-329-3p (ID 000192), mmu-miR-206-3p (ID 000510), mmu-miR-34b-3p (ID 002618), which were normalized by snoRNA202 (ID 001232). As shown in Figure 4, we could confirm the microarray expression data for the expression levels of all miRNAs validated. In the trained group at 7 days we observed only one miRNA, miR-329-3p, was slightly increased around 0.5-fold change (Fig.4A). However, other miRNAs seemed to present an increase without significant statistic. The miR-21a-5p and miR-215-5p were 0.9-fold change increased in T7, as well as miR-206-3p was 0.6-fold change (Fig.4A). The expression levels of several miRNAs were significantly increased in the trained group at day 28 when compared with sedentary animals, including miR-10a-5p, miR-21a-5p, miR-206-3p, miR-329-3p, miR-212-5p, and miR-215-5p which were presented the largest fold change (2.6, 2.6, 3.0, 2.4, 3.0 and 3.9, respectively, Fig.4B). In addition, miR-29c-5p and miR-34b-3p were

increased in T28 1.81 and 1.73-fold change respectively (Fig.4B). The PI3K pathway was related to some miRNAs as miR-29c-5p and miR-34b-3p as well as Ras and MAPK signaling pathways related to miR-10a-5p and miR-212-5p. Most of the miRNAs, miR-206-3p, miR-329-3p, miR-215-5p, and miR-21a-5p were related to MAPK and insulin pathways.

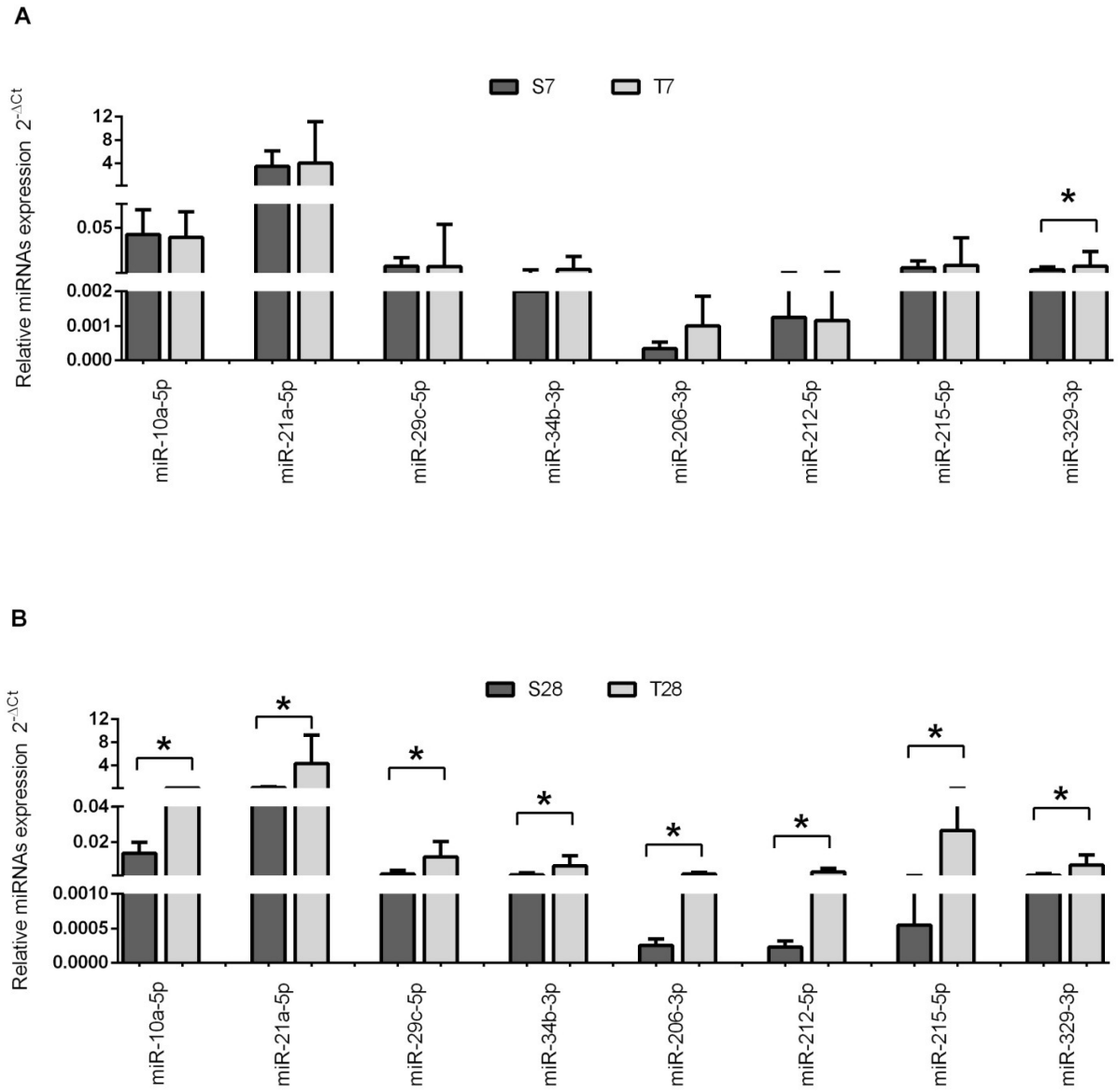


Figure 4. qRT-PCR analysis of miRNAs expression. (A) S7: sedentary at day 7; T7: trained at day 7; (B) S28: sedentary at day 28; T28: trained at day 28. N=10 each group $^{*}p<0.05$ compared to sedentary group. Data were expressed as median and interquartile range (Mann-Whitney).

We mapped the information around miRNAs which were regulated by target genes involved in signaling pathways by KEGG analysis. Figure 5 presented, as a restricted way, the miRNAs and related target genes which participate in the MAPK and Insulin in these chosen pathways to discuss. Up-regulated miRNAs were presented by red color and down-regulated miRNAs were presented as blue color. Additionally, the diamond shape presented a quantity of the genes connected to each miRNA and respective pathway.

The network presented 13 miRNAs related to of the MAPK and insulin pathways. Among these, 7 miRNAs were validated in this experiment. All of the up-regulated miRNAs validated seem to be connected with MAPK, insulin or both pathways except miR29c-5p. According to the network miRNAs were connected in these pathways by target genes which can have a validated or predicted interaction (Fig.5).

The miR-329-3p and miR-2861, up-regulated and down-regulated miRNA respectively, were shown as miRNAs connected with most of the target genes related to both pathways. However, miR-329-3p has been four common target genes of the MAPK and insulin pathways. Among validated miRNAs, miR-10a-5p, miR-212-5p, miR-21a-5p, and miR-215-5p presented only one miRNA in common between MAPK and insulin pathways. Moreover, only miR-10a-5p was connected to a validated interaction about target gene *Crkl.*

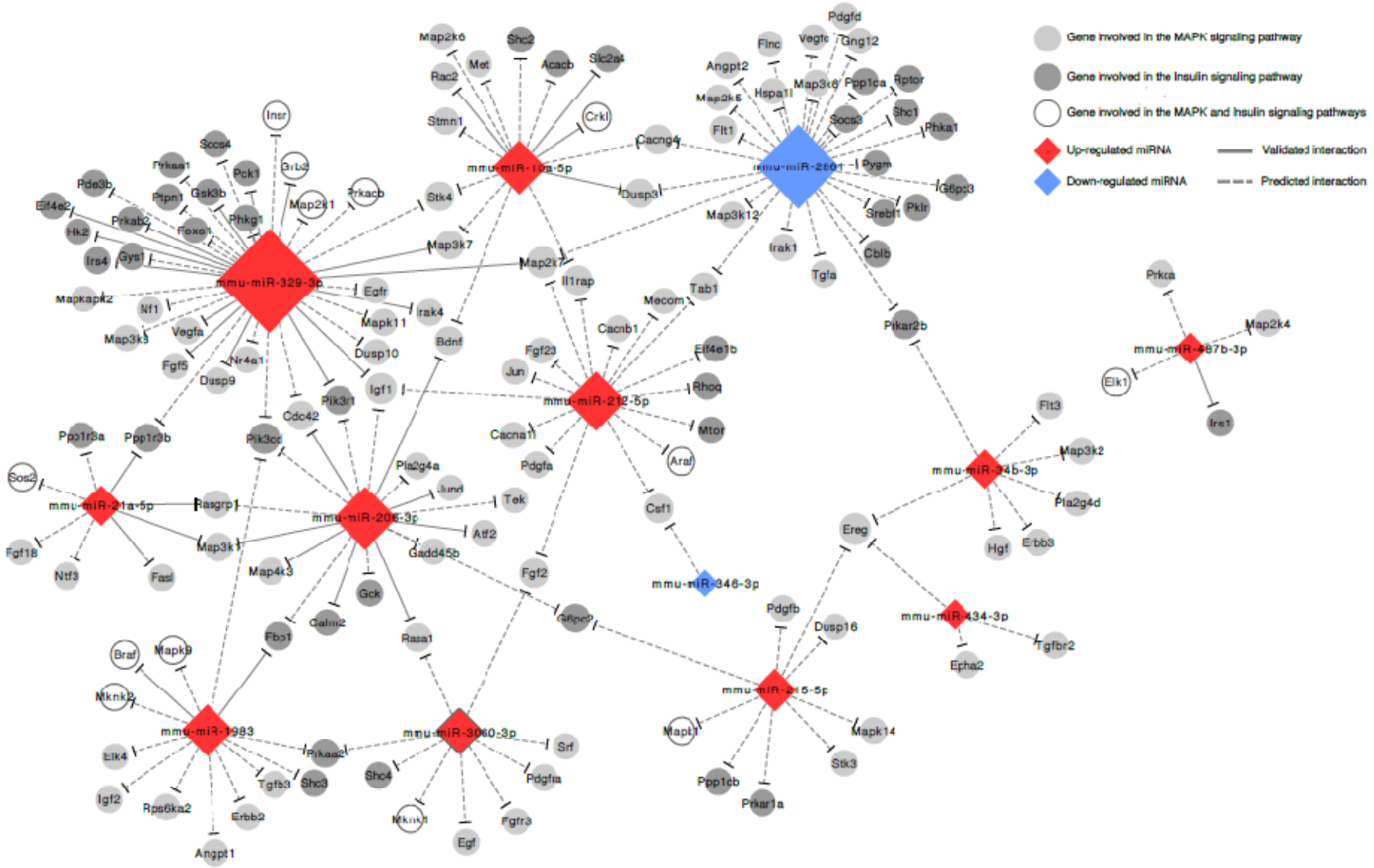


Figure 5. Interactions between miRNAs and target genes. Analysis from differentially expressed miRNAs and the restricted way the miRNAs with related target genes which participate in the MAPK and Insulin pathway. The interactions were connected miRNAs with target genes in these pathways. Up-regulated miRNAs were presented as red color and down-regulated miRNAs were presented as blue color. Additionally, the diamond shape presented a quantity of the genes connected to each miRNA and respective pathway.

Discussion

Although evidence demonstrates the cardiovascular benefits caused by exercise, the mechanisms that lead to physiologic cardiac hypertrophy involved in the heart are still unclear. miRNAs are small molecules which participate in several diseases and has been considered a cardiac regulators of gene expression [28]. Exercise is a non-pharmacological method to reduce adipocyte mass, increases insulin sensitivity, increases muscle strength and endurance, increases antioxidant levels, and increases HDL, while decreasing LDL and total triglycerides [29–31]. Concerning cardiovascular parameters, exercise reduces blood pressure, improves ejection fraction and vascular function, besides increasing angiogenesis and promotes improved cardiac muscle [32–34]. In the present study the exercise training developed physiological cardiac hypertrophy in animals trained at 7 and 28 days. The extent of cardiac hypertrophy was 9 and 13% in both groups (T7 and T28, respectively) which agrees with previous studies that showed between 7 and 29% of cardiac hypertrophy in mice [14,35,36]. The difference of the cardiac hypertrophy index between our study and others can be assigned by animal lineage differences. Zaglia et al and Liu et al achieved 22 and 29%, respectively, about cardiac hypertrophy index in C57BL/6J mice developed by swimming protocol [35,36]. Martinelli et al induced cardiac hypertrophy by wheel-running in Balb/c mice and the cardiac hypertrophy (7 and 11%) was similar to the present study [14]. We promoted cardiac hypertrophy between 9 and 13% in Balb/c mice which was supported by previous studies.

Differently from pathological cardiac hypertrophy, in physiological cardiac hypertrophy there is no fibrosis and fetal genes expression. The activation of the fetal

genes in the adult heart occurs after cardiac insults and is ubiquitously used as a biomarker of cardiac hypertrophy [37,38]. The heavy chain myosin (MHC), especially β-myosin isoform (*Myh7*), is the predominant in the developing heart of the mouse, however, it is replaced by α-myosin (*Myh6*) after birth. The change in the MHC isoform is an important process in which the heart adapts its mechanical performance and efficiency to the postnatal circulation. During cardiac hypertrophy and heart failure, the expression of *Myh7* gene is induced together with several other fetal cardiac genes. In a mouse or rat, *Myh7* is the predominant isoform in the ventricle from the fetus to the adult, but the ratio of the *Myh6* to *Myh7* is reduced in failing ventricles, as is observed in the mouse's heart [39]. Some fetal genes are regulated differently in fetal *vs.* diseased hearts. Atrial and brain natriuretic peptide (ANP and BNP) are small peptide hormones. The prohormone precursors of ANP and BNP are encoded by the *Nppa* and *Nppb* genes (atrial natriuretic peptide type A and B), respectively, and are some of the most commonly measured members of the fetal gene program [40]. The *Nppa* has distinct regulatory sequences that are activated in the embryonic heart and the adult failing heart [41]. Furthermore, fetal genes were performed to prove physiological cardiac hypertrophy. Thus, we confirmed the absence of pathological cardiac hypertrophy, since the fetal genes did not differ between groups as well as there was no presence of fibrosis in cardiac tissue corroborating previous studies [14,42].

Several differential miRNAs have been identified in physiological cardiac hypertrophy and can participate in cardiac muscle changes but whether these miRNAs were involved in determined genes or pathways that still unknown. We observed in the microarray that the miRNAs expression, which responds to changes caused by training

exercise stress, resulted in the development of physiological cardiac hypertrophy. Furthermore, we revealed that exercise regulates a large number of the up-regulated miRNAs expression. Among these miRNAs, miR-21 and miR-212 have been associated with cardiac injury and cardioprotection after ischemia; and exercise[42] besides relating to PTEN/AKT and autophagy pathways [43,44]. According to our results, the up-regulated miRNAs as miR-21a-5p and miR-212-5p were expressed in swim training seem to participate in signaling pathways to promote metabolic changes. Including insulin and MAPK signaling pathways by target genes as *Map3kq, Pp1r3a*, *Map2k7 and Mtor*. miR-212-5p has been associated with target genes related to Parkinson's Diseases, breast cancer, and other diseases [45,46]. We identified, for the first time a relation of miR-212-5p in physiological cardiac hypertrophy and *Mtor* as target gene in the insulin pathway known to participate in hypertrophy development in previous studies, however, as target of miR-99 and miR-100 [47]. Some previous studies reported that miR-21 was up-regulated in swim trained rats and myocardial disease by MAPK signaling [42,48]. Also, miR-21a-5p has been studied as mediator of the cardioprotection and participant to PTEN pathway [49,50]. In our protocol, miR-21a-5p seems to participate in physiological development by MAPK pathway genes as *Map3k1.*

The MAPK pathway contributes to skeletal muscle differentiation and myogenesis is down-regulated by myogenesis-associated miRNAS (mamiRS) due to up-regulation of miR-1a-3p, miR-133a-3p, and miR-206-3p [51]. In addition, miR-206-3p one type of miRNA specifically or highly expressed in skeletal and cardiac muscle, is known as a myomiR [52]. The myomiRs are involved during multiple biological processes including skeletal muscle growth, development, and maintenance of hypertrophy and

atrophy [52,53]. In the present study, miR-206-3p seems to regulates the majority of MAPK pathway genes besides shares with miR-21a-5p the target gene *Map3k1*. The miRNA-206 is down-regulated in type 2 diabetes mellitus, however there is no target gene described [54]. Despite this, we showed the up-regulated miR-206-3p as regulating target gene *Pik3r1* which participates in insulin signaling during swim training.

The down-regulated miR-329-3p has been associated with cervical cancer, whereas, when up-regulated, it has been described as related to cardiac amyloidosis[55,56]. We observed that miR-329-3p was up-regulated and involved in the insulin pathway with the majority of target genes connected in this miRNA such as *Foxo, IRS4*, and *Pik3r1*. Among these genes, *Pik3r1* also signalized in PI3K and mTOR pathways as we have found in physiological cardiac hypertrophy. Several slightly different versions of this regulatory subunit are produced from the *Pik3r1* gene; the most abundant of these is called *P85*α or *Pik3*α. The primary function of the subunit is to regulate the enzyme's activity, an enzyme called phosphatidylinositol 3-kinase (PI3K). Studies suggest that PI3K signaling may be involved in the regulation of several hormones, including insulin, which helps control blood sugar levels. PI3K signaling may also play a role in the maturation of fat cells (adipocytes) [57]. A recent study has revealed that the activation of PI3K/AKT/mTOR signaling axis plays a role in physiological cardiac hypertrophy by swimming of miRNAs regulation which up-regulate *Pik3*α [42]. Despite *Pik3r1* gene being validated in different sceneries, we did not find evidence as a target gene of miR-329-3p. Thus, we observed that *Pik3r1* is a possible target gene of both miR-329-3p and miR-206-3p in the insulin pathway.

miR-10a-5p seems to regulate only 3 genes related to the insulin pathway, as *Slc2a4*, and others related to MAPK pathway, for example, *Bdnf* and *Map3k7*. In cervical cancer an overexpression of miR-10a-5p suppresses division and proliferation by *Bdnf* targeting and miR-10a regulates *Map3k7* in endothelial inflammation [58,59]. On the other hand exercise promotes *Bdnf* expression due to inflammatory cytokines signalization in humans. We can suggest from these results and previous studies an interaction between miR-10a-5p and *Bdnf* target in the hypertrophic heart which can be caused by transitory cytokines stimulated in exercise. *Slc2a4* gene codifies GLUT4 protein, the major glucose transporter of brown and white adipose tissues, skeletal and cardiac muscles. Furthermore, *Slc2a4* gene becomes a promising target for pharmacogenomics of insulin resistance as reviewed Esteves et al [60] and we showed a possible regulation by miR-10a-3p after exercise. In the same line, miR-215-5p and miR-34b-3p have the largest proportion target genes related to MAPK pathway, which is known for regulation of extracellular signal and microtubule associated during survival and cellular proliferation [61]. Investigators have been focused on miRNAs which target genes associated with collagen deposition/fibrosis as miR-29. Moreover, the physiological and pathological cardiac hypertrophy can be to distinguish activation of the RAS, cardiac fibrosis and angiogenesis. The fibrosis was followed by a miR-29 reduction in myocardial infarction whereas after swimming the miR-29a and miR-29c were elevated besides being associated with down-regulation collagen genes [62]. Although our study did not identify miR-29c-5p target gene we corroborate with up-regulation profile of this miRNA in physiological cardiac hypertrophy.

Conclusion

In conclusion, in the present swimming protocol we were able to induce physiological cardiac hypertrophy, and by microarray some miRNAs were identified to be related to this process. All changes in the selected miRNAs with altered expression were confirmed by real-time PCR. The main pathways identified for most of validated miRNAs were involved MAPK and Insulin pathways. Interestingly, increased miR-329-3p was observed both at early and late times of hypertrophy (7 and 28 days), as several target genes were identified for this miRNA, suggesting for the first time a role in physiological hypertrophy. We believe that by understanding the molecular profile of miRNAs and related pathways behind the physiological cardiac stimuli, it might be possible to explore new therapeutic targets in cardiovascular diseases.

Supplementary Tables

Supplementary Table 1. Microarray samples. Individuals morphologic characteristic of animals chosen for microArray gene chip. S28: sedentary at day 28; T28: trained at day 28. N=5 and N=6, respectively.

Supplementary Table 1. Results of microarray samples chosen for GeneChip

ID	Group	LVW (mg)	TL (mm)	BW (g)	LVW/TL (mg/mm)	LVW/BW (mg/g)
16	S28	85,50	16,20	26,17	5,28	3,27
21	S28	83,90	16,44	29,76	5,10	2,82
23	T28	101,20	16,30	23,80	6,21	4,25
25	T28	107,50	16,81	26,43	6,40	4,07
26	T28	110,40	16,42	27,52	6,72	4,01
28	T28	103,60	16,06	25,65	6,45	4,04
29	T28	102,40	16,37	24,56	6,26	4,17
33	T28	101,60	16,75	27,97	6,07	3,63
48	S28	84,00	16,56	27,12	5,07	3,10
49	S28	78,00	16,68	30,47	4,68	2,56
51	S28	84,70	16,63	29,56	5,09	2,87

BW body weight, LVW left ventricular weight, TL tibial length.

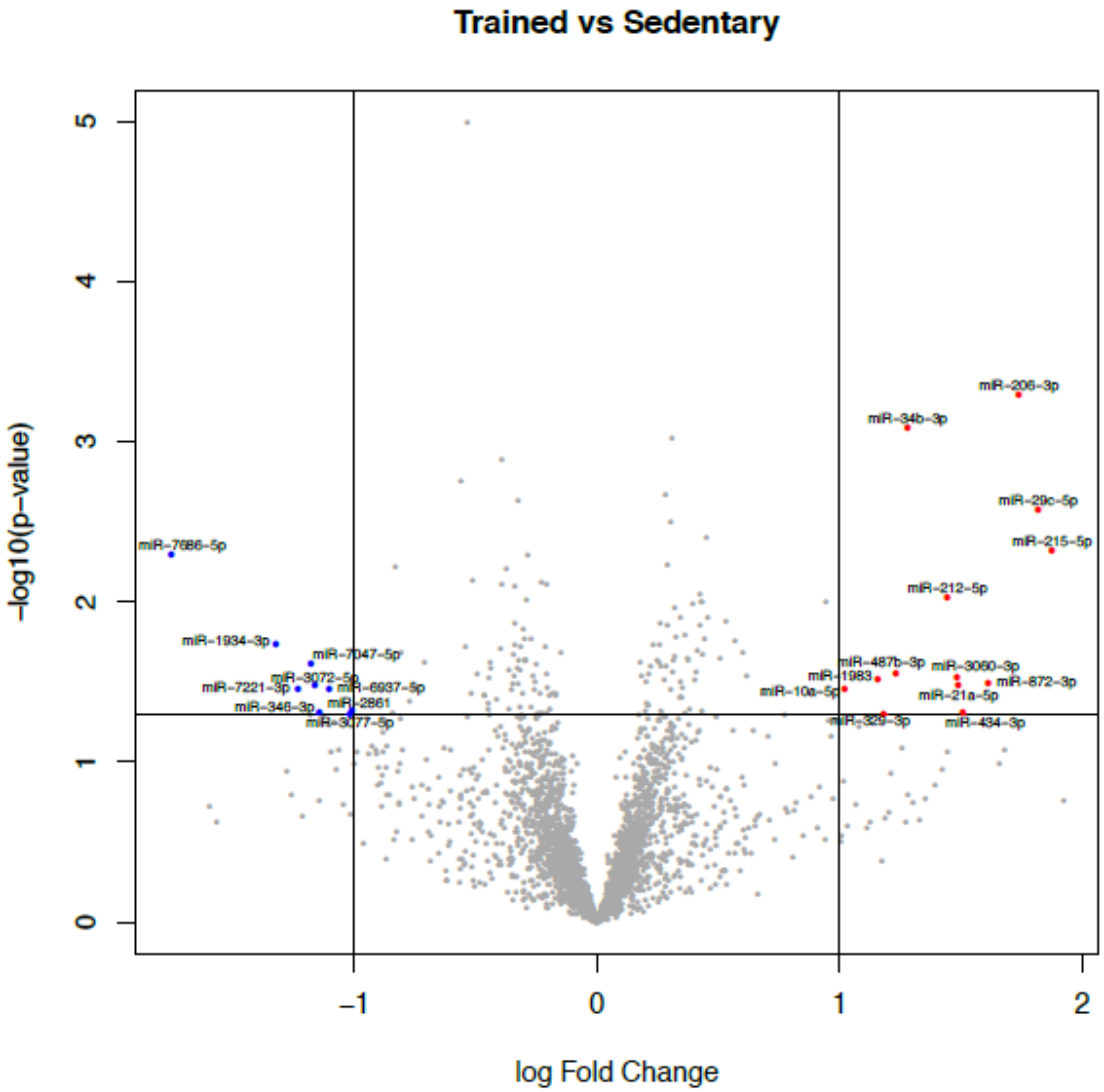
Supplementary Table 2. Differential expression analysis of microarray data. Statistics obtained for the 3163 mouse-related probes assessed using miRNA 4.0 array. P-values were estimated with Welch's test and fold changes are provided in log-scale. S28: sedentary at day 28; T28: trained at day 28. N=5 and N=6, respectively.

Supplementary Table 3. In silico analysis of target genes. List of experimental and predicted interactions retrieved in the bioinformatics analysis of up/down-regulated miRNAs, as well as the relation of miRNA regulators per target mRNA.

Supplementary Table 4. KEGG analysis. Functional enrichment analysis using KEGG Pathway database for targets of up/down-regulated miRNAs. Pathways with p- value <0.05 are listed as potentially associated with the miRNAs of interested.

Supplementary Figure

Supplementary Figure 1. Volcano Plot representation of differential expression analysis of miRNAs in trained vs. sedentary animals. Red and blue points mark the up and down-regulated miRNAs, respectively.



Supplementary Methods

Animal randomization

Animals were randomly allocated per box and lot of age and after the setting in the boxes animals were randomly allocated for cage to sedentary or exercise-training groups and were evaluated at early (7 days) or established (28 days) follow-up, thus generating four groups: S7 (sedentary 7 days, n=12), S28 (sedentary 28 days, n=12), T7 (trained 7 days, n=13), and T28 (trained 28 days, n=15). Therefore, follow the description of the allocation: the first batch of 15 animals was divided into 3 boxes at random. The animals were allocated between 5 per box. Each box received a number randomly from 1 to 3. The box 1 was chosen as sedentary animals, the box 2 and 3 were chosen as trained animals. The second batch was divided into 4 boxes to allocate 17 animals. The box 1 and 3 with 4 and 5 animals, respectively were chosen as sedentary, the box 2 and 4 with 4 animals each box were chosen as trained, respectively. The last batch was divided into 4 boxes to allocate 20 animals. As sedentary was chosen the box 1 and 3 with 5 animals each box. As trained was chosen the box 2 and 4 with 5 animals each box.

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Parte IV

Conclusões

Com os estudos aqui apresentados, concluímos que há escassez de publicações avaliando miRNAs em modelos de hipertrofia cadiaca fisiologica. Além disso, existe uma grande variabilidade com relação à expressão dos miRNAs em diferentes modelos de hipertrofia cardíaca fisiológica o que dificulta um consenso nos resultados de determinados miRNAs descritos. Mesmo assim, foi possível identificar dois miRNAs com perfil mais consistente entre os estudos avaliados. O miR-143 e miR-499 foram estudados por mais de um autor e os resultados foram semelhantes, o que direciona atenção especial para esses miRNAs no contexto da hipertrofia cardíaca fisiológica.

No segundo artigo realizamos um estudo experimental, no qual modelo de hipertrofia cardíaca fisiológica em camundongos resultou em 22 miRNAs diferencialmente expressos. Destes miRNAs oito foram escolhidos para validação e, por métodos de bioinformática, foi possível inferir vias de sinalização importantes no desenvolvimento da hipertrofia cardíaca fisiológica. As vias identificadas estavam relacionadas à sinalização pró-hipertrofia, crescimento e sobrevivência. Destacamos a via MAPK e insulinauma vez que estão fortemente envolvidas com o exercício devido a ativação ou inibição de genes preditos nessas vias. Além disso, os miRNAs identificados nesse modelo parecem participar particularmente nessas vias. Dentre os miRNAs escolhidos para a validação, sete confirmaram sua expressão como aumentada aos 28 dias. Adicionalmente, o miR-329-3p foi o único miRNA com

expressão aumentada tanto no grupo em desenvolvimento da hipertrofia cardíaca (T7) quanto no grupo de hipertrofia cardíaca estabelecida (T28). Esse estudo sugere investigar e validar os alvos identificados como preditos para os miRNAs avaliados.

Diversas diferenças foram observadas nos resultados, tanto nos artigos incluídos na revisão sistemática quanto comparando-os ao nossos resultados experimentais. O quanto esta heterogeneidade nos resultados decorre de diferenças metodológicas, ou da variabilidade biológica inerente ao modelo estudado, é algo que merece ser melhor avaliado. Diferentes metodologias para o microarrango foram identificadas, bem como heterogeneidade nos processo de validação. Dessa forma, ambos os estudos abrem uma discussão acerca da variabilidade e do envolvimento dos miRNAs na hipertrofia fisiológica, além de atentar para a importância da qualidade na apresentação dos protocolos utilizados e dos resultados encontrados.

Produção Complementar

1. Identification of candidate biomarkers for transplant rejection from transcriptome data: A systematic review. Paladini SV; **Pinto GH**; Bueno RH; Calloni R; Mendoza MR. *Submitted to Molecular Diagnosis & Therapy.*
2. Myostatin-mTOR-autophagy pathway signaling during early and late exercise-induced cardiac muscle hypertrophy. **Pinto GH**; Andrades ME; Cohen C; Martinelli NC; Clausell N; Rohde LE ; Mendoza MR ; Biolo A. *Submitted to American Journal of Physiology-Regularory, Integrative and Comparative Physiology.*
3. Cardiac hypertrophy in mice submitted to a swimming protocol: Influence of training volume and intensity on myocardial renin angiotensin system. Soares D; **Pinto GH**; Lopes A; Sturza DC; Andrades ME; Clausell N; Rohde LE; Leitão ST; Biolo A. *Submitted to American Journal of Physiology-Regularory, Integrative and Comparative Physiology.*
4. Vaporized perfluorocarbon reduces inflammatory activity during lung ischemia reperfusion injury. Ferrari RS; Solari IG; Simoneti LEL; Thomaz LDGR; Borges AM; Cohen C; **Pinto GH**; Feijo CA. *Submitted to Pulmonary Pharmacology & Therapeutics.*
5. Projeto de pesquisa: Avaliação do efeito dos produtos finais de glicação avançada sobre a autofagia no infarto agudo do miocárdio em modelo animal
6. Projeto de Pesquisa: Polimorfismo 1166A>C no gene do receptor tipo 1 da angiotensina II (AGTR1) na insuficiência cardíaca.

7. Projeto de Pesquisa: Papel dos MicroRNAs na Hipertrofia Cardíaca Fisiológica e Patológica: Expressão e Avaliação da Modulação por Espécies Reativas do Oxigênio Diferenciação entre microRNAs expressos na Hipertrofia Cardíaca Fisiológica e Patológica.
8. Projeto de Pesquisa: mitofagia na hipertrofia cardíaca em camundongos: comparação de modelo fisiológico *vs* patológico.