UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Departamento de Genética, Ecologia e Evolução
Programa de Pós-Graduação em Genética

Júlia Meireles Nogueira

CARACTERIZAÇÃO *IN VITRO* E *IN VIVO* DOS EFEITOS DO TRATAMENTO DE CÉLULAS MUSCULARES COM A MOLÉCULA ORIENTADORA POR REPULSÃO A (RGMA) DURANTE A REGENERAÇÃO MUSCULAR

Belo Horizonte

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ATA DE DEFESA DE TESE

ATA DA DEFESA DE TESE	**171/2023**
Julia Meireles Nogueira	**entrada** **2º/2018** **CPF: 099.974.446-12**

Às oito horas e trinta minutos do dia **27 de fevereiro de 2023**, reuniu-se a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Caracterização in vitro e in vivo dos efeitos do tratamento de células musculares com a Molécula Orientadora por Repulsão a (RGMa) durante a regeneração muscular "**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, a Presidente da Comissão, **Erika Cristina Jorge**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	**Instituição**	**CPF**	**Indicação**
Erika Cristina Jorge	UFMG	261.370.228-11	APROVADA
Adriana Abalen Martins Dias	UFMG	544.099.346-00	APROVADA
Albená Nunes da Silva	UFOP	787.399.856-87	APROVADA
William Antônio Gonçalves	FMRP-USP	016.560.786-60	APROVADA
Claudia dos Santos Mermelstein	UFRJ	975.150.437-68	APROVADA

Pelas indicações, a candidata foi considerada: APROVADA

O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 27 de fevereiro de 2023.

Erika Cristina Jorge

Adriana Abalen Martins Dias

Albená Nunes da Silva

William Antônio Gonçalves

Claudia dos Santos Mermelstein

Assinatura dos membros da banca examinadora:

Documento assinado eletronicamente por **William Antonio Gonçalves**, **Usuário Externo**, em 27/02/2023, às 12:32, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

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Sim, finalmente chegamos à conclusão de mais uma etapa. Se a vida fosse uma escalada de montanha, eu diria que cheguei em um dos pontos de parada para poder dar uma respirada e contemplar o caminho que já percorri até aqui. A gente chega cansado, com o pé doendo, respiração ofegante, com itens a menos na mochila e se perguntando a todo momento " o que é que eu estou fazendo aqui?"? Sim. Mas ao mesmo tempo, se a gente erguer a cabeça e perceber por onde já passamos e onde chegamos, dá um orgulho danado, né? E é claro que essa caminhada não é feita sozinha. Tem muita gente para compartilhar água, animar com uma musiquinha, parar pra oferecer o band aid pro calcanhar ralado, carregar o saco de dormir pra deixar sua mochila mais leve. E é aqui e agora que eu tenho que agradecer a todos os montanhistas que cruzaram os meus caminhos. Alguns estão por aqui desde os preparativos da viagem, uns resolveram se aventurar por outras trilhas para chegarem em pontos de parada diferentes do meu, enquanto outros começaram em pontos beeem distantes e se juntaram nos últimos quilômetros dessa caminhada. Mas saibam que todos vocês me ajudaram a ser e a conquistar o que eu tenho hoje.

A Erika é o tipo de montanhista experiente, que já passou por esses caminhos e que tem me ajudado desde que eu entrei nessa aventura doida: 12 anos atrás. Ela pegou na minha mão, me deu a primeira bota e apontou o caminho: vaai lá. Muitas das vezes eu me perdia? Voltava sem saber por qual caminho seguir? Ia para o caminho errado para ter certeza que era o errado mesmo? Muitas vezes! Mas ela celebrava comigo cada caminhada pela trilha certa, com direito a dancinha da vitória. Sempre que podia, ela dava seu jeitinho de me mostrar que era possível seguir pela trilha com a vista mais bonita, mesmo que ela tivesse que gritar o guia e pedir para me esperar amarrar o sapato para seguir adiante. E assim, ela me acompanhou durante todas essas fases: iniciação científica, intercâmbio, cursos internacionais, congressos, processos seletivos, mestrado, aulas, doutorado e continuamos contando. Muito obrigada por toda paciência, liberdade, incentivo, mudança de rota, oportunidade e carinho. Se hoje eu chego até aqui, uma pessoa muito diferente daquela que entrou no lab, foi graças a você, que me deixou percorrer muitos caminhos e encher a minha mochila com uma infinidade de apetrechos diferentes que esbarrei ao longo da minha jornada.

A Gerluza é aquela montanhista que tem a roupa mais adequada, a que sabe cozinhar a melhor refeição, a que fabrica seu próprio colchão, emite os avisos luminosos, já tem os planos B, C e D desenhados em seu caderninho de bolso e se você se machucar, ela tem o curativo, o remédio e o contato do resgate. Foi ela quem abriu as portas da sua salinha e me deu a chance de trabalhar nesse lab que desde então tem sido minha casa. E a partir daí, convivendo com essa mulher ligada no 220v, eu aprendi a me envolver, querer e participar de outras mil atividades ao mesmo tempo. Além dela ter me puxado para trilha da extensão e fazer com que eu me transformasse e me apaixonasse por essa caminhada, ela também foi a responsável por ter me dado a oportunidade de ser professora voluntária e permitir com que eu me descobrisse e passasse de “jamais serei professora, Deus me livre” para “caramba, gostei disso! Eu quero ser professora”. Gê, você que puxa a orelha com força, mas acolhe na mesma intensidade: muito obrigada por tudo o que fez e faz por mim. Se hoje eu sou avacalhada e brincalhona, pode ter certeza que aprendi com você que tudo pode ser mais leve e divertido.

Durante essa caminhada eu conheci tantos montanhistas no nosso acampamento. Eu aprendi muito, comemorei, ensinei, repeti, discuti, brinquei, experimentei, xinguei, gargalhei, chorei, cantei e vibrei. Passei por tantas fases, mudanças de dinâmica, mutirões de limpeza, bolinhos de aniversário, quebramos a cabeça para fazer as coisas funcionarem, incentivamos uns aos outros, cuidamos do acampamento, me senti em casa. O labode foi um lab escola, onde, além de aprender sobre ciência, que era o que estava no meu contrato, levo comigo lições sobre amizade, empatia, paciência, trabalho em equipe. Aos da velha guarda/dream lab: Íria, Igor, Matheus, Débora, Rayan, Copola, Aline Martins, Chicó. Aos da segunda leva: Juliano, Alinne, Bruno, Samira, Cris, Cris, Sarah. Aos jovens: Carol, Iago, Clara, Bárbara, Xulelena, Nayara, Luzia, Ricardo, Amanda. Aos novatos: Amanda, Douglas, Cristhian, Kirsty, Jade e tantos outros que passaram por ali: muito obrigada por terem sido o melhor time que poderíamos ter sido. Cada um de vocês passou e deixou um pedacinho em mim.

Eu não poderia deixar de escrever um parágrafo especial praquele que fez a minha caminhada mais incrível: Rayan! Como a gente cresceu, né meu amigo? Quem diria que aquela dupla de atrapalhados, que no início mal sabia identificar os tubinhos, chegaria a ter uma ligação tão massa? Foram tantas aventuras juntos: busca ovo,

coleta peixe, inclui embrião, faz pcr, almoça junto, compra canudinho de doce de leite, dá carona, fica confortável, faz noivado, dança igual, faz parceria científica, se comunica em idioma próprio, dá tudo de si, fica super feliz com as nossas conquistas! Eu tenho um orgulho danado de você! O que a ciência uniu, nem o lattes separa! Minha dupla para todas as horas. Obrigada por ser alegria, colo, incentivo, música, sinusite, carinho, cuidado, plenitude e belíssimo! Conte comigo para tudo nessa vida! Te amo muito.

Além do meu acampamento fixo, eu acabei visitando alguns outros grupinhos aos quais eu sou igualmente grata. Agradeço ao NAPG, por ter me recebido com tanto carinho, sob a coordenação da Profa Adriana Abalen, e ter me dado a chance de participar de várias reuniões e eventos tão legais. Com certeza foi uma outra maneira de trabalhar com pessoas de outros universos, o que contribuiu demais para enriquecer a minha caixinha de ferramentas. Agradeço a equipe atual: Glória Franco, Erika Jorge, Rose, Flavinha, Wesley, Núbia, Amandas, Vivian e Bruna. Agradeço ao projeto do PDEG coordenado pela Profa Juliana Estanislau, onde pude auxiliar na organização e produção de material didático audiovisual complementar de disciplinas do ciclo básico do ICB/UFMG e que são destinados a estudantes com deficiência ou dificuldade de aprendizado. Agradeço a galera do eterno “trem bão é ciência”, grupo formado por alunos da PPG genética que defendem a ciência e se preocuparam em fazer a divulgação científica e desmistificar o que acontece dentro dos laboratórios. Foi muito incrível conviver um pouco mais com vocês e informar a sociedade sobre tantos assuntos importantes. Agradeço a comissão organizadora do GeneTime2022, coordenado pela Profa Fernanda Antunes, Prof Fred Soriani e Prof Renato Santana. Pensar que começamos sem saber onde estávamos e entregamos um super evento com palestrantes de vários lugares do Brasil, com espaço para as crianças (filhos e filhas de cientistas) brincarem, com minucursos diversos. Sensação de dever cumprido. Isso porque nos bastidores a nossa equipe foi muito maravilhosa. Guardo cada um de vocês no coração!

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“Quem tem amigos, tem tudo”. É engraçado pensar que ao longo da nossa vida, muitas pessoas passam pelos nossos caminhos e as relações que estabelecemos com esses seres humaninhos é bem dinâmica: tem aqueles que um dia foram muito próximos, mas agora são apenas conhecidos, outros te apoiam independente de onde você esteja ou da besteira que você esteja fazendo, muitos surgem do acaso e não desgrudam mais. E que sorte a minha de ter encontrado tanta gente legal, dentro e fora da academia. A Prica veio para iluminar a minha vida. Dona de um sorriso largo, ela veio para me preencher, me ensinar, me levar para comprar brusinha, me mostrar que a vida é doce, mas não é mole, me apoiar, torcer em todas as etapas e mostrar que é possível ter 3 empregos, ser mãe, cientista, fazer atividade física e andar sempre bem vestida, de unha feita e cabelo impecável #pricaju. A Judy está comigo desde a primeira semana de aula na biologia. Desde então ela já passou por 3 cursos diferentes e hoje é a melhor médica desse universo. Ela está sempre presente, enchendo a minha bola, perguntando, acompanhando, torcendo e vibrando. A Gabs e a Babs são uma dupla que me conhecem desde que eu me entendo por gente. Essa amizade já atravessou a infância, adolescência, oceanos, pandemias e ao mesmo tempo que tudo mudou, nada parece ter mudado. Minhas roscas queriiiidas

que me apoiam sempre. A Fabianinha foi um presente que Bh me deu: chegou ali perto do início do doutorado, dividimos o mesmo teto, a cerveja, o jantar, as angustias, as conquistas, as fofocas e eu morro de orgulho dela! O meu grupinho do livro: Agatha, Flavinha, Mari, Tati e Deh. Que riqueza poder conviver com essas mulheres incríveis, fortes, fofas e amores. Com vocês eu sinto que a gente pode tudo: ao infinito e além. Obrigada por se fazerem presentes em todos os momentos.

Recentemente aumentei a minha lista de amigos ao me deparar com um pessoal que sabe que “o divertido não é o contrário de sério, o divertido é o contrário de chato”. Esse pessoal é sem limite, bagunceiro, zueiro, sério, inteligente, fofo, carinhoso e tem me feito um bem danado. Ali encontrei amizades que quero levar para a vida toda. Essa turminha é cheia de gente disposta a ajudar, incentivar, brincar, torcer, palpitar, improvisar, ensinar e se mostrar presente, apesar da distância. Ali eu posso brincar com coisa séria, desabafar diante das dificuldades, me inspirar e estar rodeada de mentes brilhantes. Eu agradeço todos os dias por ter vocês na minha vida. Quem é de verdade sabe quem é de mentira e ainda bem que a gente tem a gente: Ci (a melhor dupla do mundo e sócia do VAI QUE), Pri (a que nunca se envolve e inspiração diária), Mari (a maior detentora de frases e reflexões que conheço), Mel (doçura em forma de gente), Rogério (Gru e mestre do improviso), Sania (um presente em forma de fofura e carinho), Cici (zueira sem limites), Erica (Rainha das apresentações), Namaria (dona e proprietária de todos os ombros), Pacca (População da escrita científica), Ed (Rei do Agro), Deco (Dono dos fungos), Mauro (Reitor de tudo isso) e demais piramideiros. Vocês mudaram a minha vida! Obrigada por fazerem parte dos meus dias.

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aventura. Obrigada por ser chão, colo, sorriso, besteiradas, gargalhada! Essa conquista é nossa.

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Dito isso, me sinto pronta para continuar a minha escalada pela montanha e me preparar para as novas aventuras, encontros, desencontros e conquistas.

Resumo

O sistema muscular desempenha variadas funções relacionadas à locomoção, respiração, suporte corporal, síntese hormonal e reparo. O principal componente envolvido no reparo e desenvolvimento muscular são as células satélites, que estão inseridas em um complexo nicho composto por células intersticiais e redes vasculares e neurais. A Molécula Orientadora por Repulsão membro a (RGMa), foi inicialmente descrita durante o crescimento e migração axonal durante o desenvolvimento do sistema nervoso. RGMa também desempenha papéis em tecido não neurais, como na formação óssea, resposta imune, angiogênese e desenvolvimento do tecido muscular. Trabalhos recentes do nosso grupo de pesquisa revelaram que a RGMa é expressa em células precursoras da musculatura esquelética e que sua superexpressão induz a hiperplasia e hipertrofia em células da linhagem muscular esquelética imortalizadas. Assim, o presente trabalho buscou investigar a relação entre as células satélites e RGMa durante a regeneração muscular. No primeiro capítulo, nós observamos que RGMa é capaz de promover a hipertrofia quando injetada no músculo tibial anterior, tanto na musculatura de animais saudáveis como em um cenário de injúria muscular química causada por cloreto de bário. Juntos, esses resultados revelam possíveis estratégias terapêuticas, envolvendo a biologia molecular, para resolver ou amenizar os sintomas causados por miopatias. No segundo capítulo, nós caracterizamos os efeitos de RGMa nas células satélites *in vitro*. RGMa foi encontrada no núcleo de células satélites, provavelmente sendo transportada por BMP, uma vez que o tratamento dessas células com um inibidor de BMP, a dorsomorfina, foi capaz de inibir a localização nuclear de RGMa. Além disso, RGMa parece induzir a proliferação de células satélites, assim como sua diferenciação em um ambiente com condições favoráveis. Nossos resultados trazem novas informações relacionadas com a heterogeneidade e comportamento de células satélites cultivadas na presença de RGMa.

Palavras-chave: desenvolvimento muscular, RGMa, regeneração, célula satélite, hipertrofia

Abstract

The muscular system performs a variety of functions related to locomotion, respiration, body support, hormone synthesis, and repair. The main component involved in muscle repair and development are satellite cells, which are embedded in a complex niche composed of interstitial cells and vascular and neural networks. The Repulsive Guidance Molecule member a (RGMa) was initially described during axonal growth and migration during nervous system development. RGMa also plays roles in non-neural tissue, such as in bone formation, immune response, angiogenesis, and muscle tissue development. Recent work by our research group revealed that RGMa is expressed in skeletal muscle precursor cells and that its overexpression induces hyperplasia and hypertrophy in immortalized skeletal muscle cells. Thus, the present work sought to investigate the relationship between satellite cells and RGMa during muscle regeneration. In the first chapter, we observed that RGMa is capable of promoting hypertrophy when injected into the tibialis anterior muscle, both in the musculature of healthy animals and in a scenario of chemical muscle injury caused by barium chloride. Together, these results reveal possible therapeutic strategies, involving molecular biology, to resolve or alleviate symptoms caused by myopathies. In the second chapter, we characterize the effects of RGMa on satellite cells *in vitro*. RGMa was found in the nucleus of satellite cells, probably being transported by BMP, as the treatment of these cells with a BMP inhibitor, dorsomorphine, was able to inhibit the nuclear localization of RGMa. Furthermore, RGMa seems to induce the proliferation of satellite cells, as well as their differentiation in an environment with favorable conditions. Our results bring new information related to the heterogeneity and behavior of satellite cells cultured in the presence of RGMa.

Keywords: muscle development, RGMa, regeneration, satellite cell, hypertrophy

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Capítulo 2

Estrutura da tese

Este documento foi elaborado com uma breve introdução para a identificação da proposta e relevância do tema, seguida pelos objetivos gerais e específicos. Os demais itens serão apresentados na forma de dois capítulos, escritos em formato de artigos científicos, sendo eles:

Capítulo 1: Trabalho submetido na revista International Journal of Molecular Science, para ser publicado na edição especial “Emerging Mechanisms for Skeletal Muscle Mass Regulation” intitulado como “Intramuscular injection of Repulsive Guidance Molecule a (RGMa) recombinant protein induces hypertrophy in health and regenerating muscle”, que se refere aos resultados obtidos pelas análises realizadas *in vivo*.

Capítulo 2: Trabalho em execução intitulado provisoriamente como “RGMa is expressed in activated satellite cells nuclei and is associated with cell proliferation”, que se refere aos resultados obtidos pelos experimentos realizados *in vitro*.

Para finalizar, serão apresentados os trabalhos complementares desenvolvidos até a conclusão desta tese.

Sumário

1. Introdução

O sistema muscular é um dos tecidos mais abundantes do corpo. Em indivíduos saudáveis, ele pode ser responsável por 40 a 50% do peso corporal. Além de ser essencial para promover atividade locomotora, a musculatura esquelética também é encarregada pelo suporte postural, respiração, metabolismo e síntese hormonal (Kistner et al., 2022; Bawa et al., 2021; Yamakawa et al., 2020). As unidades celulares básicas responsáveis pela contração muscular são as miofibras, formadas durante o desenvolvimento embrionário e fetal e, em algumas espécies, até a vida pós-natal (Sanger et al., 2010; Ochi & Westerfield, 2007; Srinivas et al., 2007).

1.1 A origem da célula muscular estriada esquelética: a miogênese

Durante o desenvolvimento embrionário, as células musculares estriadas esqueléticas têm origem do folheto germinativo conhecido como mesoderma, particularmente do mesoderma paraxial ou dorsal.

O mesoderma paraxial, localizado em ambos os lados do tubo neural e da notocorda, passa pelo processo de segmentação e epitelização, para dar origem a uma estrutura embrionária conhecida como somitos. Inicialmente, os somitos são estruturas cilíndricas e epiteliais. Com o desenvolvimento, no entanto, estas estruturas epiteliais se compartimentalizam e se diferenciam em duas regiões: (i) o dermomiótomo da porção mais dorsolateral da estrutura e o (ii) esclerótomo, que se forma na região mais ventromedial dos somitos (Brent & Tabin, 2002; Applebaum & Kalcheim 2015). Em seguida, surgem mais dois compartimentos nos somitos: (iii) o miótomo, que se origina a partir da migração ventral de células das extremidades do dermomiótomo e o (iv) sindétomo, que se estabelece a partir da migração dorsal de células do esclerótomo (Della Gaspera, Weill & Chanoine, 2022; Della Gaspera et al., 2019; Buckingham & Rigby, 2014; Shi & Garry, 2006; Gros et al., 2005). Cada um desses compartimentos fica responsável pela formação de um tecido/estrutura no corpo do embrião: (i) o dermomiótomo formará a derme da pele e o miótomo; (ii) o esclerótomo formará as vértebras e costelas; o (iii) miótomo formará a musculatura estriada esquelética; e o (iv) sindétomo formará os tendões que unem a musculatura esquelética às vértebras (Scaal, 2016; Lepper & Fan, 2010; Brent & Tabin, 2004; Brent et al., 2003; Hirsinger et al, 2001; Tajbakhsh et al., 1997).

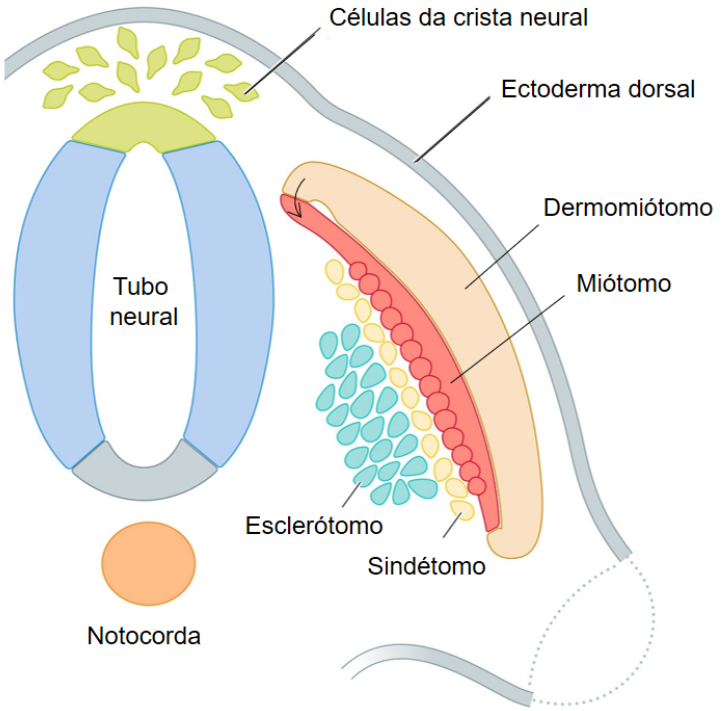

Figura 1. Processo de segmentação dos somitos. As extremidades do dermomiótomo participam da formação do miótomo. As células musculares progenitoras derivam da porção central do dermomiótomo. Essas células migram diretamente para o miótomo, onde permanecem como mioblastos em proliferação e são responsáveis pelo crescimento do miótomo. Adaptado de Choi et al., 2020.

Nos miótomos, as células precursoras musculares apresentam alta capacidade proliferativa e expansiva. Sendo assim, a habilidade de se delaminar tanto do dermomiótomo quanto do miótomo permitem a sua migração e atuação durante a miogênese embrionária e fetal em diferentes regiões do organismo (Relaix et al., 2005; Kassar-Duchossoy et al., 2005; Birchmeier & Brohmann, 2000). Os processos de somitogênese e miogênese, que envolvem intensa dispersão e diferenciação celulares, são regulados por sinalizações moleculares provenientes de estruturas presentes nas regiões adjacentes aos somitos (Sambasivan & Tajbakhsh, 2007; Tajbakhsh & Cossu, 1997). A migração e a especificação celular estão fortemente envolvidas com a expressão de moléculas como Notch (Buas & Kadesch, 2010), Fgf (Pownall & Isaacs, 2010), Wnt (von Maltzahn, Chang, Bentzinger, & Rudnicki, 2012), Bmp (Pourquie et al., 1996; Amthor et al., 1999), Shh (Brent et al., 2002; Johnson & Tabin, 1995; Fran & Tessier-Lavigne, 1994) e de fatores miogênicos como Myf5, MyoD, MyoG e MRF4 (Chal & Pourquie, 2017; Hubaud & Pourquie, 2014; Bentzinger, Wang, & Rudnicki, 2012; Pownall, Gustafsson, & Emerson, 2002) (Figura 2).

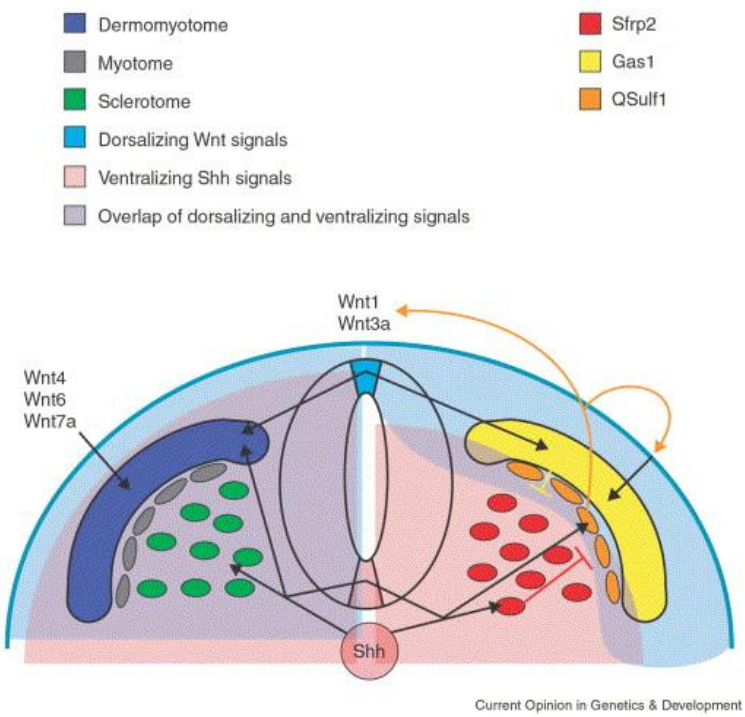

Figura 2. Localização de sinalização durante a padronização dorsoventral. Sinalização molecular durante a compartimentalização dos somitos (Brent & Tabin, 2002)

A musculatura estriada esquelética do tronco é estabelecida em diferentes etapas, conhecidas como ondas miogênicas, que marcam diferentes fases de migração de precursores musculares e formação de miofibras (Figura 3). A primeira onda ocorre quando precursores musculares, localizados no dermomiótomo e que inicialmente expressam os fatores Pax3 e Pax7, deixam o ciclo celular e passam a expressar fatores miogênicos: na porção central do dermomiótomo, as células expressam Myf5, MyoD e Mrf4, enquanto as células das extremidades do dermomiótomo expressam, MyoD e níveis moderados de MyoG e Mrf4 (Della Gaspera et al., 2012). Os primeiros mioblastos embrionários vão compor o miótomo primário, que será formado por células alongadas que se proliferam ao longo do miótomo e contribuem para a formação de fibras primárias multinucleadas (Kahane et al. 2007; Gros et al., 2005; Hollway et al., 2003). Essa primeira etapa migratória servirá de plataforma para que a proliferação de mioblastos fetais aconteça assim como a fusão de miócitos e resulte na formação de miofibras secundárias, que vão contribuir para o crescimento e formação do miótomo fetal (Messina & Cossu, 2009; Cossu & Biressi, 2005). Essa etapa é caracterizada pelo crescimento e desenvolvimento do tecido muscular, assim como sua inervação. As miofibras formadas por um sincício alongado e

multinucleado, derivado de fusões de miócitos, apresentam mionúcleos periféricos, são envoltas por uma membrana basal e possuem seu citoplasma altamente organizado (Chal & Pourquie, 2017). Esse citoplasma é composto por miofibrilas, que são grupamentos de proteínas, constituído, principalmente por actina, miosina, troponina e tropomiosina, que desempenham a função de contração e relaxamento da musculatura esquelética (Sweeney & Hammers, 2018). A terceira onda miogênica é caracterizada pela delaminação das células progenitoras proliferativas da porção central do dermomiótomo e, assim, essas células, que também são denominadas de células satélites, vão atuar durante o desenvolvimento fetal e pós-natal e permitir o crescimento da musculatura esquelética após o nascimento (Ben-Yair and Kalcheim 2005; Gros et al. 2005; Kassar-Duchossoy et al. 2005; Relaix et al. 2005; Ben-Yair et al. 2003; Kahane et al. 2001).

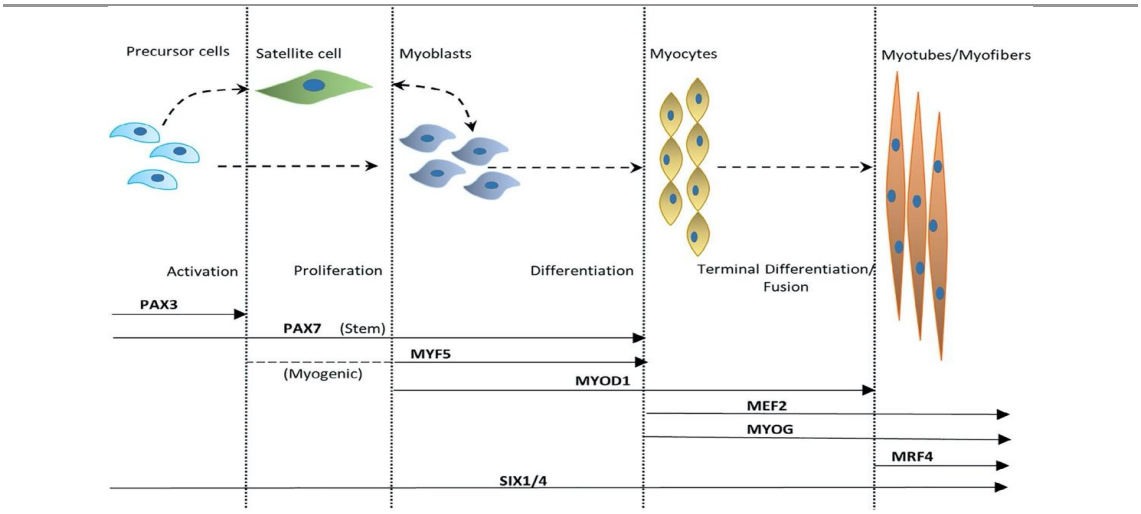

Figura 3. Fatores de transcrição envolvidos na regulação da miogênese. Células satélites expressam Pax7 e assim que são ativadas iniciam a expressão de Myf5 (Mukund & Subramaniam 2020).

Ao final do desenvolvimento pós-natal, as células satélites deixam o ciclo celular, se tornam as células tronco adultas da musculatura esquelética, se localizam entre a lâmina basal e a membrana das miofibras e entram em quiescência (Gattazzo et al., 2020; Bachman et al., 2018; Brohl et al., 2012; White et al., 2010; Relaix et al., 2005; Kassar-Duchossoy et al., 2005; Mauro, 1961). Durante o período pós-natal, as miofibras mantêm a capacidade de se regenerarem após injúria muscular, graças à presença dessas células satélites, as precursoras da miogênese (Charge & Rudnicki, 2004).

1.2 A miofibra madura

As células musculares maduras possuem uma alta concentração de proteínas especializadas que utilizam a energia química de moléculas de ATP para gerar força mecânica em forma de contração muscular (Sweeney & Hammers, 2018). Os sarcômeros são as unidades contráteis básicas da musculatura esquelética e se dispõem em série, no citoplasma das miofibrilas (Figura 4). As bandas Z, delimitam o início e o término de um sarcômero. A banda A localiza-se na porção medial do sarcômero, onde os filamentos grossos, ou miosinas, estão alinhados (Craig, 2004).

Entre as bandas Z e a banda A está a banda I, composta pelos filamentos finos de actina e suas proteínas associadas: tropomiosina e troponina, que respondem a íons cálcio para regular a contração da musculatura esquelética (Sweeney & Hammers, 2018). O deslizamento entre os filamentos de actina e miosina resultam em uma aproximação entre as bandas Z e assim resultam na contração de toda a unidade muscular.

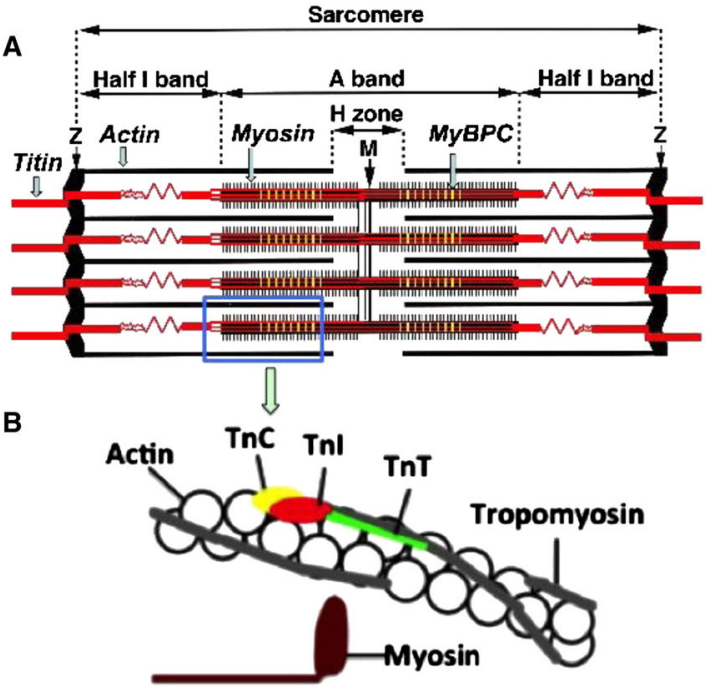

Figura 4. Sarcômero como unidade básica de contração muscular. Actina, miosina, tropomiosina e complexo de troponinas compõem as principais proteínas contráteis das miofibrilas (Yin et al., 2015)

Dentre os componentes responsáveis pela contração muscular, as miosinas, que são os filamentos grossos, podem apresentar-se como diferentes isoformas. Basicamente, as fibras chamadas de lentas apresentam grande concentração de

mioglobinas, mitocôndrias e dessa forma apresentam maior resistência à fadiga. Por outro lado, as fibras rápidas, apresentam menor concentração de mioglobinas e mitocôndrias e uma grande concentração de enzimas glicolíticas (Schiaffino, 2018). Parâmetros bioquímicos e velocidade de contração são as principais características que diferem as fibras rápidas das lentas. As fibras do tipo 2A, 2B e 2X, expressam as miosinas MYH2, MYH1 e MYH4, respectivamente; e são abundantes nos músculos de contração rápida. Já as miofibras do tipo 1, que expressam MYH7, são majoritariamente presentes nos músculos lentos (Agarwal et al., 2020).

Além das miofibras, que são os componentes contráteis, a musculatura esquelética também apresenta outros elementos envolvidos com o suporte, nutrição, comunicação e renovação desse tecido. O sistema vascular garante o aporte nutricional, trocas gasosas e remoção de metabólitos tóxicos, assim como as células do sistema imune que realizam a fagocitose de debris celulares provenientes de eventos apoptóticos e atuam na fase inflamatória frente à miotraumas (Fuchs & Blau, 2020; Verma et al., 2018). A comunicação entre o sistema nervoso e o sistema muscular acontece através dos prolongamentos de motoneurônios, que formam as junções neuromusculares essenciais para o processamento da informação contrátil. Além disso, os fibroblastos presentes entre as miofibras são responsáveis pela produção de matriz extracelular e as células tronco musculares, ou células satélites, contribuem para a regeneração da musculatura esquelética (Zhang et al., 2021; Plikus et al., 2021; Hortells et al., 2019).

1.3 As células-tronco musculares adultas: as células satélites

As células satélites possuem a mesma origem das células musculares: os precursores musculares se proliferam e migram do dermomiótomo para o miótomo e durante esse processo, as células podem de dividir por dois planos de divisão celular: o assimétrico e o simétrico (Rodriquez-Outeirino et al., 2021; Feige et al., 2021). Durante as divisões assimétricas, uma célula mãe dá origem à duas células filhas com funções diferentes: uma fica responsável por manter a população de célula tronco e a outra fica comprometida com a linhagem muscular ao migrar para o miótomo, se diferenciar em mioblasto e se comprometer com a diferenciação muscular (Cossu & Tajbakhsh, 2007; Motohashi & Asakura, 2014). E é por isso que as células satélites são consideradas células tronco: elas têm a capacidade de se auto renovarem e podem se diferenciar em diferentes tipos celulares, dependendo do

estímulo e nicho em que ela é inserida. Já na divisão simétrica, uma célula mãe dá origem a duas células filhas idênticas, que possuem as mesmas funções e que podem estar relacionadas com a manutenção da população de células tronco, em prol do aumento do número de células comprometidas com a linhagem muscular.

Em mamíferos, o músculo esquelético apresenta grande capacidade de regeneração, quando comparado ao tecido muscular liso e cardíaco (Sweeney & Hammers, 2018; Holban et al., 2016). O sucesso da regeneração depende da extensão e da natureza da lesão e o processo regenerativo é composto por três etapas básicas: (i) de inflamação, (ii) regeneração e (iii) remodelamento e reparo do tecido muscular (Muire et al., 2020). Alguns fatores de crescimento já foram identificados atuando na função das células satélites. Os receptores e ligantes da superfamília TGFβ, por exemplo, controlam o crescimento muscular, a ativação das células progenitoras, fibrose e formação de tecido adiposo e ósseo ectópico (Glass, 2010; Serrano et al., 2011; Sartori et al., 2014). As Proteínas Morfogenéticas Ósseas (*Bone Morphonegetic Protein*, BMP), um sub-grupo da superfamília TGFβ, foram inicialmente descritas como potentes inibidoras da diferenciação muscular (Amthor et al., 2004; Frank et al., 2006), embora baixos níveis de BMP sejam capazes de induzir a expressão de Pax3 na miogênese primária (Amthor et al., 1998; 1999). Mais recentemente, trabalhos mostraram efeitos positivos das BMPs na proliferação de células satélites e progenitores musculares fetais (Chen et al., 2021; Wang et al., 2010) além da atuação sobre o ganho de massa muscular (Sartori et al., 2021; Sandri et al., 2013).

1.4 A Molécula Orientadora por Repulsão a (*Repulsive Guidance Molecule a*, RGMa)

A molécula orientadora por repulsão a (*Repulsive Guidance Molecule member a*, RGMa) compõe uma família de moléculas orientadoras (RGMa-d) responsáveis pelo crescimento e migração de axônios. Suas funções foram inicialmente descritas em tecidos neurais (Müller et al., 1996; Monnier et al., 2002; Schwab et al., 2005), sendo associada aos mecanismos de sobrevivência e diferenciação de neurônios (Matsunaga & Chédotal, 2004; Matsunaga, 2006; Koeberle et al., 2010); enquanto o bloqueio de sua ação via tratamento com anticorpo estabilizados em áreas de lesão

da medula espinhal demonstrou ser eficaz na indução da regeneração neuronal (Hata et al., 2006; Doya et al., 2006).

Estudos funcionais mais recentes vêm revelando novas funções das RGMs em tecidos não-neurais e nem sempre associados à função original de moléculas orientadoras de axônios. RGMc ou Hfe2 foi associada ao controle da homeostase de ferro (Taranath et al, 2020; Papanikolaou et al., 2004); e mutações na sequência deste gene foram associadas com a doença Hemocromatose Juvenil (Hernández et al, 2021; Papanikolaou et al., 2004). RGMa foi encontrada atuando na formação dos ossos (Lu et al, 2019; Zhou et al., 2010); na resposta imune (Mothe et al, 2022; Nohra et al., 2010; Muramatsu et al., 2011); e na formação de vasos sanguíneos (Tang et al, 2022; Harada et al., 2016). RGMb foi associada ao desenvolvimento de testículos (Xia et al., 2005); à proteção de células renais contra injúria (Liu et al., 2016) e à formação de cistos nos rins (Liu et al., 2016). Juntos, estes trabalhos sugerem que o papel biológico que as RGMs exercem nos organismos é muito mais amplo, não se restringindo ao de moléculas orientadoras de axônios.

As RGMs sinalizam nas células via (i) o receptor Neogenina (Kee et al, 2008); e (ii) como correceptores da via de sinalização BMP (Corradini et al, 2009; Samad et al., 2005; Babitt et al., 2006). Além de sinalizar utilizando pelo menos uma das vias já associadas ao potencial de proliferação da população de células satélites (BMP), algumas evidências reveladas por nosso grupo de pesquisa vêm sugerindo um papel para RGMa também na origem, proliferação e diferenciação de células musculares esqueléticas (Jorge et al., 2012; Martins et al., 2015; Copola et al., 2021; Costa et al., 2022). O padrão de expressão de RGMa durante a embriogênese de galinha indicou a presença de transcritos para esta molécula nas células musculares pioneiras no dermomiótomo dos somitos, em sobreposição ao local de expressão Pax7, que é marcador das precursoras das CS (Jorge et al., 2012). Estudos *in vitro* vem demonstrando ainda um papel para RGMa no controle do tamanho e do mecanismo de fusão de mioblastos em miotubos multinucleados. A super-expressão de RGMa *in vitro* promoveu o aparecimento de células mais largas, hipertróficas, em sua maioria miotubos multinucleados; enquanto o *knockdown* de RGMa resultou em células menores, atróficas, com morfologia típica de mioblastos indiferenciados (Martins et al., 2015). A super-expressão de RGMa em mioblastos resultou ainda em um aumento de três vezes no índice de fusão das células transfectadas, comparadas ao

controle (Martins et al., 2015). Resultados semelhantes foram ainda obtidos com o uso de RGMa na forma de proteína recombinante, com efeitos surpreendentes na indução da proliferação de mioblastos, mesmo em condições de cultivo indutores de diferenciação miogênica (Costa et al., 2021).

O presente trabalho teve como objetivo (i) investigar as possíveis funções de RGMa na proliferação de células satélites isoladas (ii) e avaliar os efeitos da injeção intramuscular de RGMa durante a regeneração muscular em camundongos. Este trabalho sugere a identificação de RGMa como um fator indutor da proliferação de células-tronco musculares, com potencial para gerar alvos de inovação para o desenvolvimento de terapias gênicas ou celulares voltados ao tratamento de miopatias como na Distrofia Muscular de Duchenne e de diversas outras doenças neurodegenerativas com o objetivo de reverter o fenótipo muscular atrófico.

2. Objetivo Geral

Investigar os efeitos da Molécula Orientadora por Repulsão membro a (RGMa) em células satélites durante a regeneração e crescimento do tecido muscular.

2.1 Objetivos específicos:

Capítulo 1

Investigar os efeitos da injeção intramuscular de RGMa recombinante na musculatura esquelética em animais saudáveis;

Analisar o potencial regenerativo da injeção intramuscular de RGMa recombinante na musculatura esquelética de animais com lesão muscular.

Capítulo 2

Localizar a expressão de RGMa tanto na fibra muscular isolada da musculatura de camundongos selvagens quanto nas células satélites já aderidas à placa de cultura;

Caracterizar os efeitos de RGMa sobre a proliferação de células satélites cultivadas;

Investigar se o tratamento com Dorsomorfina, um inibidor da via de sinalização BMP, é capaz de interferir na expressão de RGMa nas células satélites *in vitro*.

3. Capítulo 1

Article

Intramuscular injection of Repulsive Guidance Molecule a (RGMa) recombinant protein induces hypertrophy in health and regenerating muscle

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Abstract: Repulsive Guidance Molecule member a (RGMa) is axon guidance molecule that also play roles in non-neuronal tissues. During *in vitro* myogenesis, RGMa has consistently been found inducing nuclei accretion during myoblast differentiation into multinucleated myotubes and hypertrophy. The current work revealed that RGMa works *in vivo* as observed *in vitro*: RGMa recombinant protein intramuscular injection promoted a hypertrophic phenotype in tibialis anterior muscle, both in wild type musculature and after a chemical injury caused by $BaCl_2$ injection. We also investigated the molecular mechanisms that are induced by RGMa during *in vivo* muscle hypertrophy and we found a possible association of RGMa with the upregulation of mTOR signaling pathway. These results can open a wild field about developing new therapeutic strategies to solve or soften the symptoms of muscle disorders.

Keywords: skeletal muscle; hypertrophy; recombinant RGMa; muscle injury; muscle regeneration.

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1. Introduction

Skeletal muscle is one of the largest tissues in the human body and its weight is responsible for about 40% of the body mass [1,2]. Skeletal muscle plays critical roles in voluntary movement and has several other actions such as postural behavior, breathing and also metabolic and endocrine functions [3,4].

Skeletal muscle tissue has an innate repair mechanism which allows it to recover from injuries in daily life, such as the mechanical trauma, thermal stress, ischemia, myotoxic agents, exercise, neurological damage and other pathogenic conditions [5]. Disorderly muscle contraction process, involving communication failures between the muscular structure and the extracellular matrix, sustain an increased fragility in the sarcolemma. Hence, the influx of extracellular calcium leads to the activation of proteases, pro-inflammatory cytokines and mitochondrial dysfunction that undergoes muscle degradation and necrosis [6–9].

Muscle degeneration is followed by the activation of muscle repair process and the main actor involved in this scene are the muscle stem

cells, named satellite cells. The niche occupied by satellite cells is composed by a diversity of components [10,11]. Upon imbalance caused by exercise, injury or disease, the different types of interstitial cell and vascular and neural networks are activated and work together to ensure muscle homeostasis [12–14].

Activated satellite cell is able to fuse to a pre-existent myofiber to contribute with new nuclei to the fibers or fuse with other mononucleated myogenic precursors to form new fibers [15,16]. This satellite cell addition to skeletal muscle fibers positively promotes hypertrophy [17] and simultaneously increase myofiber cross sectional area [18,19].

Repulsive guidance molecules (RGMs) were firstly described during the neural development of chicken embryos [20] and its functions were associated with neuron survival and differentiation [21–23]. Despite being discovered acting on the nervous system, RGMa also displays functions in non-neural tissues, presenting roles during bone formation [24], immune response [25,26] and in angiogenesis [27].

Our research group has been investigating RGMa in the context of myogenesis [28–31]. *in vitro* results have been consistently showing that RGMa can induce skeletal muscle hypertrophy and nuclei accretion during differentiation, once it acts in increasing cell size and supporting fusion mechanisms of myoblasts into myotubes [29–31]. In this work we investigated the effects of the intramuscular injection of RGMa as a recombinant protein into both health and injured adult skeletal muscle.

2. Results

2.1. **RGMa recombinant protein dose-effect curve for intramuscular injection**

We first established a dose-effect curve for RGMa recombinant protein intramuscular injection. For this, 0.5, 1 and 2.5 μg of RGMa recombinant protein were directly injected into the TA muscle of 7-weeks-old mice and its effects were evaluated after 7 days post-injection (dpi), based on morphometry analysis.

At 7 dpi, we could not observe any difference in muscle weight between treated and control muscles, with none of the doses used in this work (Figures 1A-B). The analysis of the CSA revealed no difference between the TA muscle treated with 0.5 μg of RGMa protein, compared to the control (Figure 1C), which was corroborated by the analyzes of the histogram of frequency distribution (Figure 1D). A significative decrease in fiber CSA was observed with the injection of 1 μg of RGMa protein at 7 dpi, compared to the control (Figure 1E). The histogram of frequency distribution showed an increase in the frequency of smaller fibers, especially those ranging from 300 to 600 μm2, compared to the control (Figure 1F). An even smaller decrease in CSA was observed when 2.5 μg of the protein was injected, compared to the control (Figure 1G), also confirmed by the histogram of frequency (Figure 1H).

Altogether, the dose-effect curve suggested 1mg of RGMa recombinant protein as the minimum dose to induce morphological effects on the skeletal muscle cell size.

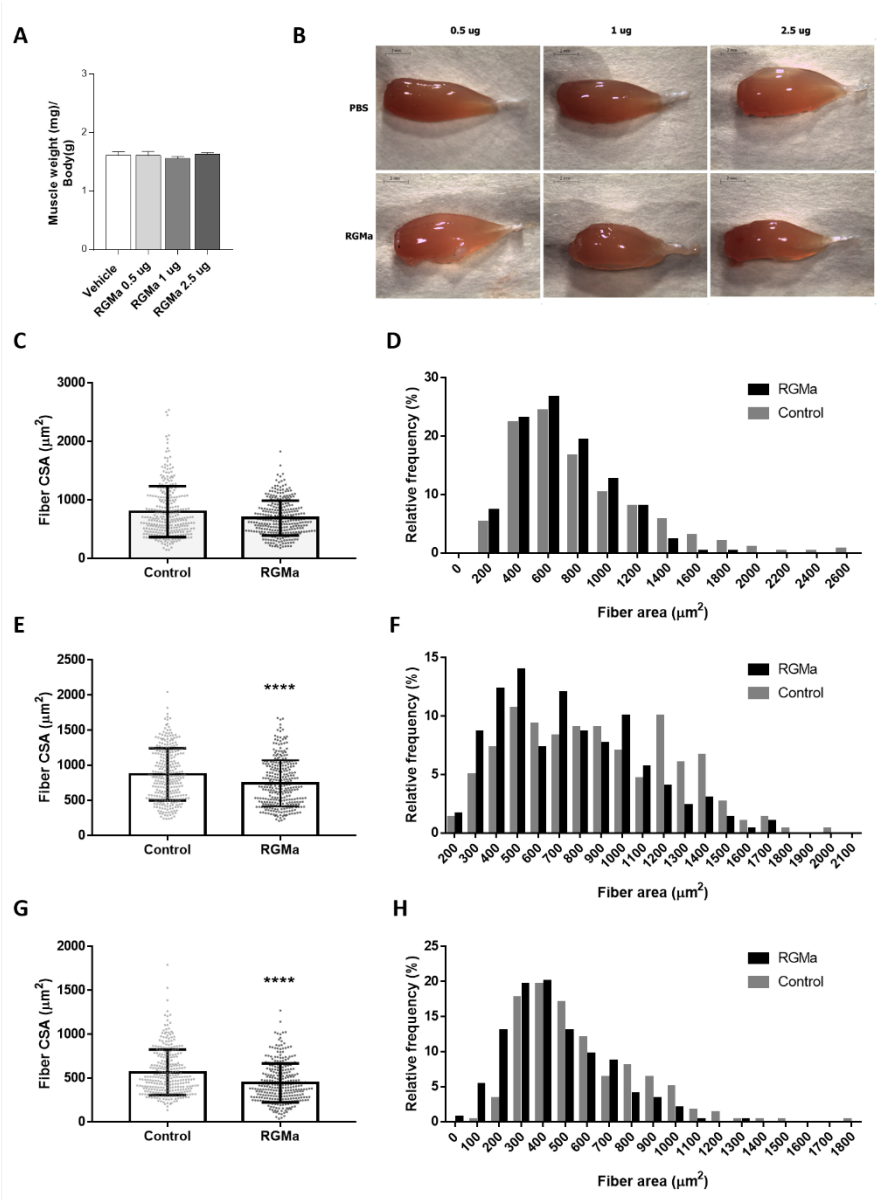

Figure 1. RGMa recombinant protein dose-effect curve for intramuscular injection. **A.** Muscle weight (mg)/body weight (g) ratio at 7 days post PBS and RGMa injection. Tibialis anterior muscle and total body were weighted in every animal. **B.** Representative images of tibialis anterior (TA) muscle from intramuscular injection of PBS and RGMa after 7 days. Scale bar: 2mm. **C.** Muscle fiber cross-sectional area (CSA) after 7 days of 0,5 µg of RGMa recombinant injection. **D.** Frequency distribution of myofiber CSA from PBS and 0,5 µg RGMa injection at 7 days post injection. **E.** Muscle fiber cross-sectional area (CSA) after 7 days of 1,0 µg of RGMa recombinant injection. **F.** Frequency distribution of myofiber CSA from PBS and 1,0 µg RGMa injection at 7 days post injection. **G.** Muscle fiber cross-sectional area (CSA) after 7 days of 2,5 µg of RGMa recombinant injection. **H.** Frequency distribution of myofiber CSA from PBS and 2,5 µg RGMa injection at 7 days post injection. Differences between groups were assessed for significance using unpaired t-test. **** $p<0.0001$. Data are presented as mean± SD.

2.2. ***Morphometrically effects of RGMa recombinant protein intramuscular injection***

To address whether RGMa protein was capable of stimulating productive muscular hypertrophy *in vivo*, 1 mg of RGMa recombinant protein was directly injected into TA muscle of 7-weeks-old mice and its effects were evaluated based on morphometry analysis, after 14 and 28

dpi, using the TA muscle from the contralateral leg injected with PBS as control (Figure 2).

After 14 dpi, morphometrical analysis revealed that the TA muscles administrated with RGMa presented smaller fiber cross sectional area (Figure 2A and B), higher concentration of small fibers (Figure 2C) and of the number of muscle cells per 100 μm2 (Figure 2D), all compared to the control TA muscle.

After 28 dpi, however, the opposite effect was observed: TA muscles treated with RGMa recombinant protein presented larger fiber cross sectional area (Figures 2E and F), higher concentration of large fibers (Figure 2G) and smaller number of cells per 100 μm2 (Figure 2H), compared to the control muscle.

These results suggested that a single administration of RGMa recombinant protein can induce effects in health skeletal muscle cell size *in vivo*.

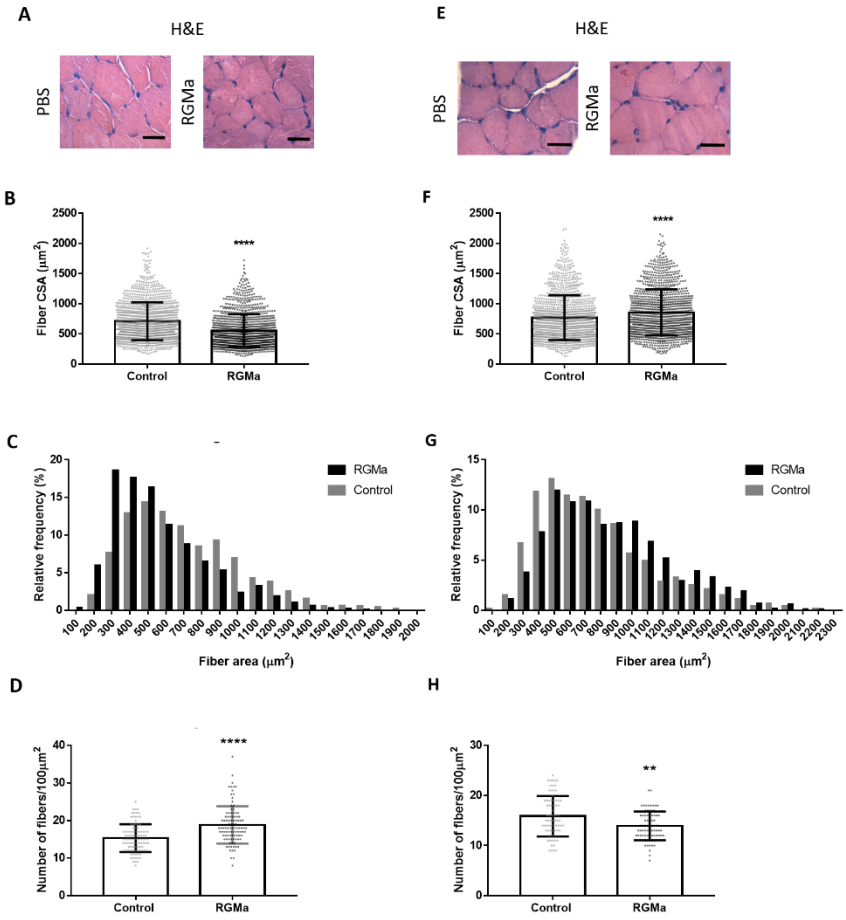

Figure 2. Recombinant RGMa injection induced muscle hypertrophy at 28 days. A. Representative images of tibialis anterior (TA) muscle sections stained with hematoxylin and eosin from intramuscular injection of PBS and RGM after 14 days. Scale bar: 20μm. B. Muscle fiber cross-sectional area (CSA) after 14 days of RGMa treatment. C. Frequency distribution of myofiber CSA from PBS and RGMa injection at 14 days post treatment. D. Number of muscles fibers counted per 100um2 in the control and treated sample. E. Representative images of tibialis anterior (TA) muscle sections stained with hematoxylin and eosin from intramuscular injection of PBS and RGM after 28 days. Scale bar: 20μm. F. Muscle fiber cross-sectional area (CSA) after 28 days of RGMa treatment. G. Frequency distribution of myofiber CSA from PBS and RGMa injection at 28 days post treatment. H. Number of muscles fibers counted per 100um2 in the control and treated sample. Differences between groups were assessed for significance using unpaired t-test. ** $p< 0.01$ and **** $p<0.0001$. Data are presented as mean± SD.

2.3. ***Gene expression analysis of myogenic and inflammatory markers***

We then inspected the possible mechanisms of action of the administration of this axon guidance molecule in the *in vivo* muscle, using qPCR. We investigated the expression of the myogenic markers MyoD and MyoG, of the hypertrophic marker mTOR, of pre-inflammatory (IL6, TNF-a) and anti-inflammatory (IL10) markers and assessed muscle fiber type using primers for Myh7 (slow, type I), Myh2 (fast, type 2A), Myh1 (fast, type 2X) and Myh4 (fast, type 2B). Relative expression analysis was performed after 14 and 28 dpi, comparing injected x control muscle (Figure 3).

Our results showed no statistic differences in the expression of MyoD and MyoG at 14 dpi, compared to contralateral muscle (Figure 3A). After 28 dpi, MyoG was found to be upregulated in the RGMa-administrated muscle, compared to the control, while no difference in MyoD expression could be observed (Figure 3B). We also found that the expression of mTOR was downregulated in the RGMa-administrated muscle, compared to the control at 14 dpi (Figure 3A), but at 28 dpi, the expression of this hypertrophic marker was found to be upregulated in the RGMa-administrated muscle, compared to the control (Figure 3B).

Related to the inflammatory markers, we found the downregulation of the expression of IL10 in the RGMa-administrated muscle at 14 dpi, compared to the control (Figure 3C), while no differences could be observed in the expression of IL6 and TNF-a, compared to the control muscle (Figure 3C). At 28 dpi, however, no effects of RGMa-administration on inflammatory markers could be more observed (Figure 3D).

RGMa administration could also modulate the expression of fiber type genes. After 14 dpi, no statistic differences were observed in the expression of any of the specific myosins (Figure 3E). However, after 28 dpi, we could note the upregulation of the Myh1 in the RGMa administrated muscle, compared to the control (Figure 3F).

Altogether, gene expression results suggested that RGMa-administration can affect (i) the expression of the differentiation (MyoG) and hypertrophic markers (mTOR), (ii) regulate the anti-inflammatory response based on IL10 expression, and (iii) the pattern of fiber types in the adult health muscle.

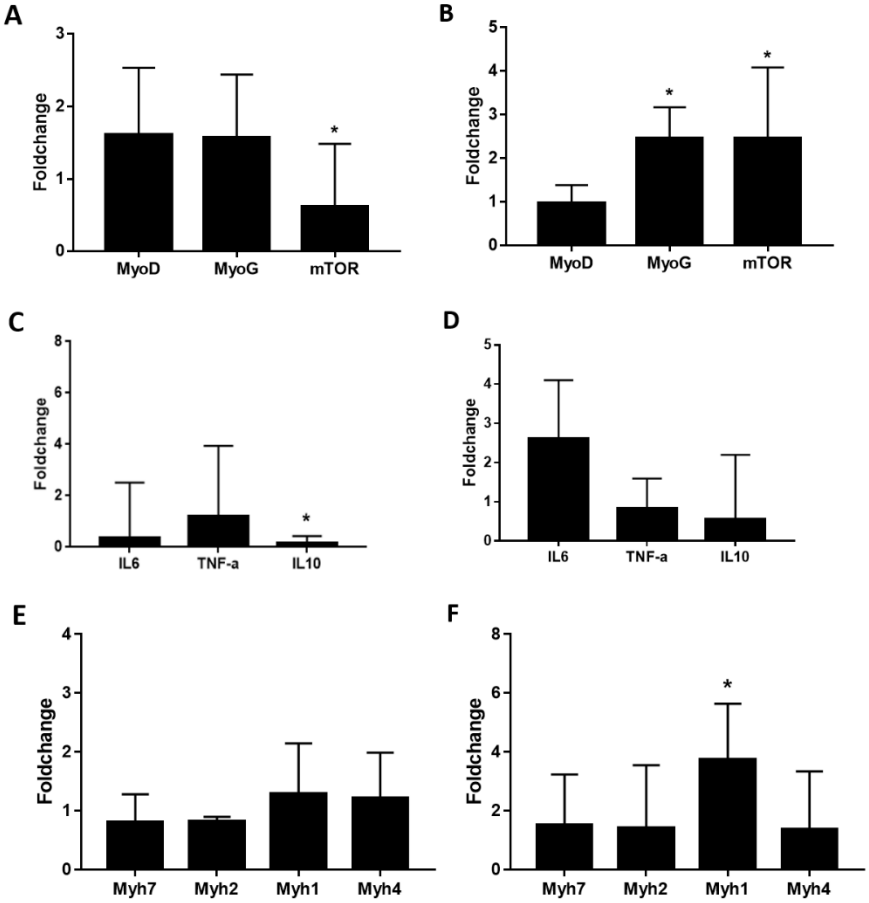

Figure 3. Gene expression involved in muscle metabolism after recombinant RGMa intramuscular injection. qPCR analysis for MyoD, MyoG, mTOR, IL6, TNF-α, IL10, Myh7, Myh2, Myh1 and Myh4 expression in freshly isolated tibialis anterior muscle from PBS and RGMa injection after (A, C and E)14th and (B, D and F)28th day of PBS and RGMa injection. Beta-actin was used as a housekeeping gene. Statistical analyses were performed using the Pairwise Fixed Allocation Randomization Test in the Rest software.

2.4. ***Intramuscular injection of RGMa recombinant protein in TA injured muscle***

Once a single RGMa intramuscular injection was able to induce hypertrophy in a health musculature after 28 dpi, we decided to investigate whether RGMa recombinant protein could induce similar effects if administrated in an injured muscle. In this work, muscle injury was induced by the intramuscular injection of BaCl2 in the TA muscles of both hindlimbs of adult mice. After four days of of BaCl2 injection, RGMa was administrated to the right TA muscle and, as a control, the left hindlimbs were injected with PBS. Morphometric analysis was performed after 2, 14 and 28 dpi (Figure 4).

At 2 dpi, transversal sections from both control and RGMa-administrated muscles showed muscle fibers with central nuclei (Figure 4A), suggesting that both muscles were affected by the injection of BaCl2 and were in regeneration process. We could detect larger fiber cross sectional area of injured RGMa-administrated muscles after 2 dpi, compared to the control (Figure 4B). This data was corroborated with the observation of the presence of higher concentration of large fibers (Figure 4C) and smaller number of cells per 100 μm2 (Figure 4D), compared to the control.

Similar hypertrophic morphological effects were also observed after 14- (Figures 4E-H) and 28-days (Figures 4I-L) of RGMa administration, always compared to the respective control muscle. The histogram of frequency distribution (Figures 4G and K) and the number of fibers per 100 μm2 (Figures 4H and L) could always confirm the hypertrophic phenotype.

All together, these results reveal that a single injection of RGMa recombinant protein can improve skeletal muscle regeneration after injury.

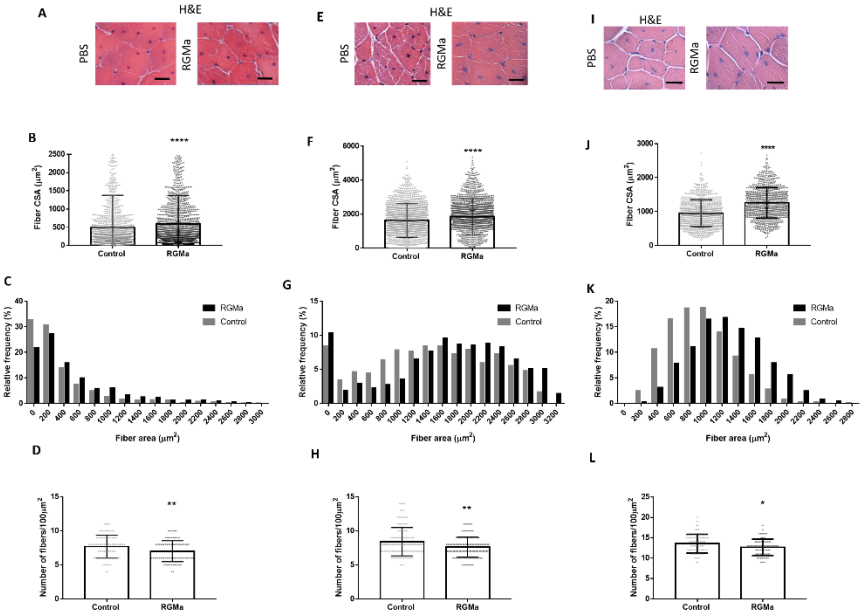

Figure 4. Muscle injury generated by BaCl2 induced greater fibers after 2 days of RGMa treatment. A, E and I. Representative images of tibialis anterior (TA) muscle sections stained with hematoxylin and eosin from intramuscular injection of PBS and RGM after 2,14 and 28 days of muscle regeneration. Scale bar: 20μm. B, F and J. Muscle fiber cross-sectional area (CSA) after 2, 14 and 28 days of RGMa treatment. C, G and K. Frequency distribution of myofiber CSA from PBS and RGMa injection at 2, 14 and 28 days post treatment. D, H and L. Number of muscles fibers counted per 100um2 in the control and treated samples at 2, 14 and 28 days post treatment. Differences between groups were assessed for significance using unpaired t-test. * p<0.1, ** p< 0.01 and **** p<0.0001. Data are presented as mean± SD.

2.5. ***Gene expression analysis during muscle regeneration***

The expression pattern of the same markers was obtained by qPCR after barium chloride injury after 2-, 14- and 28-days of RGMa administration in TA muscles (Figure 5).

Our results showed no statistic differences in the expression of MyoD after 2, 14 and 28 dpi, compared to contralateral muscle (Figure 5A, B and C, respectively). A significative effect could be observed after 28 dpi for the expression of MyoG, which was found to be downregutated in the RGMa-administrated muscle, compared to control TA muscle (Figure 5C). We could also observe effects of the RGMa-treatment in the mTOR expression, which was found to be upregulated at 14 and 28 dpi, compared to the control (Figures 5B and C). No significative differences could be observed in the expression of inflammatory markers after 2, 14 or 28 dpi, compared to the control muscle (Figure 5D-F).

Related to the fiber type genes, we could not note any significative difference after 2 dpi, compared to the control (Figure 5G). Curiously,

after 14 dpi, we could observe the downregulation of the expression of Myh4 in the lesion scenario, compared to the control muscle (Figure 5H). At 28 dpi, we found the downregulation of Myh1 and the upregulation of Myh7 expressions in the RGMa-administrated muscle, compared to the control (Figure 5I).

Altogether, these results suggested that RGMa recombinant protein administration can improve muscle regeneration and shuffle the composition of fiber types during muscle remodeling after injury.

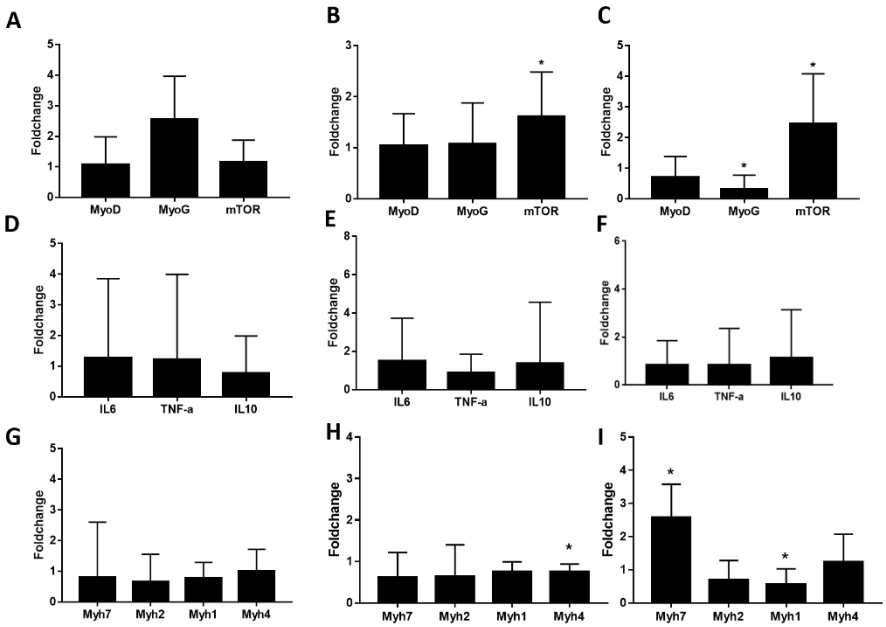

Figure 5. Gene expression involved in muscle metabolism after recombinant RGMa intramuscular injection. qPCR analysis for MyoD, MyoG, mTOR, IL6, TNF-α, IL10, Myh7, Myh2, Myh1 and Myh4 expression in freshly isolated tibialis anterior muscle from PBS and RGMa injection after (A, D and G) 2, (B, E and H) 14 and (C, F and I) 28 days of PBS and RGMa injection. Beta-actin was used as a housekeeping gene. Statistical analyses were performed using the Pairwise Fixed Allocation Randomization Test in the Rest software.

3. Discussion

The present work investigated the biological effects and the mechanisms activated after the intramuscular injection of RGMa recombinant protein. RGMa is an axon guidance molecule found to play different roles in neuronal and also non-neuronal cell types. In skeletal muscle cells, RGMa is detected in the sarcolemma and sarcoplasm and it was associated with cell hypertrophy and nuclei accretion when administrated to these cell type in culture [29–31]. Here we investigated whether RGMa could induce similar effects if directly injected as a recombinant protein into both health and injured Tibialis Anterior of adult mice.

We first established a dose-effect curve in order to determine the minimum dose of the RGMa recombinant protein that would induce an effect in a health skeletal muscle after 7 dpi. We decided to use three different doses (0.5, 1 and 2.5 μg) following what it has been used for this protein in other tissues, mainly in neural tissues [35,36]. We found that 1 μg of the recombinant protein was enough to induce an effect in the health TA muscle, even though the effect was the appearance of a number of small fibers, compared to the control TA muscle injected with the vehicle. Since the analysis was performed only after 7 dpi, we could not

determine if the presence of small fibers was a result of the induction of the molecular mechanisms that lead (i) to protein degradation in the existing fibers or (ii) hyperplasia to produce more fibers, or (iii) both.

We then injected RGMa recombinant protein in health TA muscle to evaluate its effects later post injection. We found that the muscle that received a single dose of RGMa recombinant protein kept presenting small fibers after 14 dpi, compared to the control. However, after 28 dpi, we could note that the cells from the treated muscle presented a hypertrophic phenotype, as we found cells with larger CSA than in the control. Altogether, these results suggested that the direct administration of a single dose of RGMa recombinant protein into the health muscle can affect skeletal muscle cell size *in vivo*.

The morphometrical effects of RGMa *in vivo* muscular administration were similar to those observed *in vitro*: RGMa-treatment or overexpression in C2C12 cells induce nuclear accretion in myotubes (determined by the fusion index) and also the formation of larger cells (hypertrophic phenotype), respectively [29,31]. Although being capable of inducing similar effects in skeletal muscle cell size both *in vitro* and *in vivo*, the molecular mechanisms that are modulated by RGMa in this particular tissue are not fully known. For this reason, we assessed gene expression of particular markers to try to find a clue of the pathway that could be responsible for the effects of this axon guidance molecule in the *in vivo* muscle.

Gene expression analysis revealed that RGMa treatment into health muscle could regulate the expressions of *mTOR* and the anti-inflammatory marker *IL10* after 14 dpi and upregulate the expression of *MyoG*, *mTOR* and *Myh1* after 28 dpi, always compared to the correspondent TA muscle from contralateral leg injected with PBS.

mTOR is one of the major players in controlling muscle mass and fiber size (revised by [37]. mTOR deficient mice show reduced postnatal growth due to the reduced size of fast muscle fibers, and a progressive muscular dystrophy phenotype [38]. RGMa effects on mTOR expression had been suggested in a previous work from our group [30]. In this work, RGMa effects on mTOR expression in health muscle corroborated with the morphometrical analysis: mTOR expression was downregulated after 14 dpi in the RGMa administrated muscle, compared to the control, a stage that showed the presence of small muscle fibers after RGMa injection; while its upregulation could be observed at 28 dpi, exactly when a hypertrophic phenotype was observed in the RGMa administrated muscle. These results suggest an important RGMa mechanism of action during the induction of muscle hypertrophy.

Inflammatory markers were tested in this work since RGMa has been found to exert functions in immune system [39,40] and inflammation is known to influence skeletal muscle regeneration, since a transient increase in local inflammation signaling triggers the myogenic signaling cascade to induce muscle repair, remodeling, and maintenance in healthy muscle (revised by [41]. We could find that the RGMa-treated muscle presents a downregulation of the expression of the anti-inflammatory IL10 marker. While pro-inflammatory cytokines produced by M1 macrophages (such as IL6 and TNF, for example) stimulate myoblast proliferation, anti-inflammatory cytokines from M2 macrophages (such as IL10) are known to promote their differentiation [41,42]. In this sense, RGMa would be regulating IL10 expression, interfering with the complete differentiation of the recently formed new fibers. This hypothesis can be corroborated with the effect of the treatment with this axon guidance molecule in Myogenin expression.

Curiously, Myogenin is essential for myocyte fusion and its depletion leads to formation of mononucleated myofibers [43], which is a similar effect observed after the manipulation of the RGMa expression in myoblasts in culture [29,31].

We have also investigated if the treatment with RGMa could induce any changes in the composition of the fiber types of the TA muscle, since other axon guidance molecules were found to play this role [44,45]. Tibialis anterior is essentially a fast muscle, containing a superficial region mainly composed of type-IIB fibers and a more profound region rich in IIX and IIA fibers [46,47]. Each muscle fiber type expresses one myosin isoform, being Myh7 predominant in the slow fiber type I, Myh2 in the fast type IIA, Myh1 in the fast IIX and Myh4 in the fast IIB [48]. We found that RGMa-treatment could induce the expression of *Myh1* in the TA-treated muscle after 28 dpi, compared to the control. Since we could observe the presence of larger fibers in the TA treated muscle only at this moment of the analysis, our result suggests that RGMa is inducing the IIX phenotype in the newly formed fibers, a subtype of fast-swich fiber that is already in bounty in TA muscle.

We then tested whether RGMa could induce similar effect in an injured muscle, here promoted by the injection of barium chloride directly into the TA muscle. The injury of skeletal muscle with chemical agents such barium chloride initiates a cascade of events leading to the muscle regeneration [49] and it has been used as an accessible alternative model to study muscle regeneration [2,50,51]. The healing of an injured muscle is known to follow three phases: (i) the inflammatory phase, which is established from 0 to 7 days post-injury (dpi); followed by (ii) the regeneration phase, starting from 4 to 14 dpi and (iii) the remodeling and repair phase, from 14 until 28 dpi [52]. Muscle injury initiates following hematoma formation [7,53], which is going to guarantee that the repair occurs only within the injury site.

In this model of injured muscle, a single dose of RGMa recombinant protein could consistently induce muscle hypertrophy from 2 dpi, maintaining this effect at 14- and 28 dpi. These results suggest that RGMa mechanisms of action are even more evident in the lesion context, producing larger fibers right after 2 dpi.

Gene expression analysis revealed that *mTOR* expression was upregulated in the RGMa-treated muscle at 14 and 28 dpi, both compared to their respective controls. However, *Myogenin* was found to be downregulated in the RGMa-treated muscle at 28 dpi. We could not observe any difference in the expression of the inflammatory markers comparing RGMa-treated x control muscle in any evaluated stages.

Skeletal muscle fiber type composition has already been associated with muscle disease, like dystrophies and sarcopenia [54]. In some cases, the strategy is to stimulate factors evolved in muscle dynamics and that are able to change the fiber type either from slow to fast, of fast to slow [54–56]. Another procedure to reprogram muscle constitutions is changing the neural inputs and stimulate the fibers to swift from fast-to slow or vice versa [57]. Sciatic nerve denervation is one of the most popular strategies to study skeletal muscle atrophy [58]. Besides the loss of contractile force, this model stimulates the clinical outcome of motor neuron disease or injuries that affect the muscle morphology and function [59–61]. The denervation atrophy usually regulates the slow-to-fast fiber type conversion [62,63]first affecting fast fibers, once they show greater plasticity when compared to slow fibers [64]. Considering a long-term denervation of soleus, which is considered a slow muscle, only residual slow myosin is present while fast myosin is current in the

majority of musculature. In consequence of repeated cycles of cell death and regeneration, a severe denervation of soleus resulted in a slow-to-fast transformation fiber type [62,63,65].

The vast majority of fibers in TA muscle corresponds to type 2, which configure fast fibers. After 14 dpi, the expression of *Myh4*, which characterizes the 2B fast fiber, was downregulated. In a similar way, after 28 dpi, the expression of *Myh1*, which characterizes the 2X fast fiber was inhibited, while the type 1 slow fiber expressing *Myh7* was upregulated, suggesting that RGMa, in addition to promoting hypertrophy, is capable of changing the fiber type of fast to slow in a chemical injury scenario. Tatsumi and co-workers [66] demonstrated that Sema3A works after a muscle injury, impacting the generation of slow fibers *in vivo*. i*n vitro*, Sema3a increased the formation of slow fibers during myotube development, activating signals that inhibit the expression of fast myosin. These results demonstrated that, like Sema3A, RGMa is a repulsive guidance molecule and can contribute to the formation of slow fibers after muscle injury and also is capable of inducing a hypertrophic phenotype in the tibialis anterior musculature.

4. Materials and Methods

4.1 Animals

Wild-type C57BL/6J male mice from 6- to 8-weeks old were obtained from CEBIO and maintenance and experimentation were performed according to the ethical protocol approved at UFMG (366/2019). All mice were maintained on standard conditions at a constant temperature of 24°C under an artificial 12h light and 12h dark cycle, with ad libitum access to water and food, at the animal house on Morphology Department/UFMG.

4.2 Dose-effect curve for RGMa recombinant protein intramuscular injection

A dose-effect curve was firstly established to determine the minimum dose of the RGMa recombinant protein necessary to obtain morphometrical effects after 7 days post injection (dpi). The selected doses of the mouse RGMa recombinant protein (R&D Systems) for the establishment of a curve were: 0.5 mg (injected as 50 ml of a 0.01 mg/ml solution), 1 mg (injected as 50 ml of a 0.02 mg/ml solution) and 2.5 mg (injected as 50 ml of a 0.05 mg/ml solution). For the intramuscular injection, animals were anesthetized with ketamine/xylazine. The TA muscle from the right hindlimb of each animal (n=3) was injected with RGMa recombinant protein, using PBS on the contralateral TA muscle from the hindlimb as experimental control (n=3).

4.3 Morphometric analysis

After 7-, 14-, and 28-days post injection (dpi), animals were weighed and deeply anesthetized with ketamine/xylazine. The TA muscle from the control and treated paws were harvested, weighted, and fixed in 4% PFA for 4h at room temperature.

After dehydration in an ascending series of alcohols (70%, 80%, 90%, 95% 2X), samples were embedded in paraffin and 5 μm thick sections were obtained using a Histo-Line Laboratories' MRS3500 microtome. Sections from muscle samples were stained with Haematoxylin and Eosin (HE) to determine both the Ferret diameter and the cross-sectional area (CSA) of individual myofibers. Images were acquired using the

Olympus BX50 microscope with Motic®AE31 capture system. The number of myofibers was counted from five squares of 100 mm2 each, draw and positioned in each photo using ImageJ software.

4.4 BaCl2 muscle injury

Skeletal muscle injury was induced in wild type male 7-weeks-old mice (n=3), following the protocol used by Morton [32]. Briefly, the animals were anesthetized with intraperitoneal injection of ketamine/xylazine solution (0,2 ml/20 g). The skin of each hindlimb was shaved and 1.2% BaCl2 solution dissolved in phosphate buffered saline (PBS) was injected into the TA muscle from both legs, using an insulin syringe. After four days of the BaCl2 injection, the established best dose for RGMa recombinant protein was injected, as previously described. After 2-, 14-, and 28-dpi, animals were deeply anesthetized with ketamine/xylazine (0,6 ml/20 g) and both right and left TA muscles were harvested, weighted and processed for morphometric analysis.

4.5 Real-time quantitative PCR

Total RNA was isolated from TA muscle from control and treated hindlimbs, using Tri-Reagent, following manufacturer's instructions (Sigma-Aldrich). RNA integrity was monitored by electrophoresis. 1 mg of total RNA was converted in cDNA using High-capacity cDNA reverse transcription kit instructions (Thermo Fisher Scientific). All RT-qPCR reactions were performed using a Rotor-Gene RT-PCR system (Qiagen). Each reaction contained 1x iTaq Universal SYBR Green Supermix (Bio-Rad) and 0.4–0.6 μM of primers and cDNA diluted 1:10, for a final volume of 10 μl. Primers of reference and target genes were designed using Primer3Plus software to allow the amplification of ~200 bp each. Primer sequences used in this work are as follow: GAPDH (AGGTCGGTGTGAACGGATTTG and TGTAGACCATGTAGTTGAGGTCA), MyoD (GTGGCAGCGAGCACTACA and GACACAGCCGCACTCTTC), MyoG (TGAGAGAGAAGGGGGAGGAG and CGGTATCATCAGCACAGGAG), mTOR (CGTCAATCCAGAGCACAATC and ATTTCACAATCGGAGGCAAC) IL6 (TTCCATCCAGTTGCCTTCTT and TTTCTCATTTCCACGATTTCC), TNF-α (AGGCACTCCCCCAAAAGA and CAGTAGACAGAAGAGCGTGGTG), IL10 (CAACATACTGCTAACCGACTCCT and GCCTGGGGCATCACTTCTAC), Myh1 (AGCCACTTCAGCCCAAATC and CTCGCTCTTCTCCTTCTCCA), Myh2 (AAGCCCACTTCTCCCTCATC and CCCTTCTTCTTGGCACCTTT), Myh4 (GGAGCGGATGAAGAAGAACA and TCCTCGGTCTGGTAGGTGAG) and Myh7 (CTGAGACGGAGAATGGCAAG and GTTGACGGTGACGCAGAAG). Samples were run in triplicate using the following thermal cycle conditions: 95°C for 2 min, followed by 45 cycles of a three-step reaction, denaturation at 94°C for 15 sec, annealing at 60–62°C for 15 sec and elongation at 72°C for 20 sec and finally an additional extension at 72°C for 5 min. The dissociation step was performed at the end of the amplification step to allow the identification of the specific melting temperature for each primer set. Relative gene expression was calculated using the REST 2009 software [33,34], normalized againist the expression of *gapdh*.

4.6 Statistical analysis

Statistical analysis was evaluated using the unpaired Student's t-test and represented the mean± standard deviation. The graphics were plotted using the program Prism software 7.0 version. Results were considered significant at $p<0.05$ (*), $p<0.01$ (**) and $p<0,001$ (***).

5. Conclusions

RGMa injection into health or in a chemically injured tibialis anterior muscle resulted in a hypertrophic phenotype, probably by inducing mTOR signaling. However, its action in health and injured muscle stimulates muscle fibers in different ways: in the health muscle, RGMa promoted the upregulation of fast 2X fiber type, while in the lesion scenario, RGMa inhibited the expression of Myh1, which characterizes type 2X fibers and favored the increased expression of slow fibers, which express myosin Myh7. More studies investigating the signaling pathway activated by RGMa are needed to understand and develop a therapeutic strategy involving RGMa to reverse myopathies phenotype and symptoms.

Author Contributions: J.M.N and E.C.J designed the experiments. J.M.N and A.C.C performed the experiments. J.M.N and C.D.A.A analyzed the data. J.M.N and E.C.J wrote the manuscript.

For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, J.M.N. and E.C.J.; methodology, J.M.N and A.C.C.; formal analysis, J.M.N and C.D.A.A.; writing—original draft preparation, J.M.N; writing—review and editing, E.C.J.; supervision and funding acquisition E.C.J.; project administration, J.M.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais (366/2019).

Informed Consent Statement: Not applicable

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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4. Capítulo 2

RGMa is expressed in activated satellite cells nuclei and is associated with cell proliferation

ABSTRACT

Repulsive Guidance Molecule member a (RGMa) was firstly described during the growth and migration of neurons over the development of nervous system. RGMa also play roles in non-neural tissue, such as bone formation, immune response, angiogenesis and skeletal muscle development. Previous works from our research group revealed RGMa expression in the skeletal muscle precursor cells and its overexpression induced hypertrophy and hyperplasia of a muscle cell immortalized lineage. In the current work we characterized the effects of RGMa in satellite cells, the skeletal muscle stem cells and the main actor during the development and repair of muscle damage. We found that RGMa is present in the satellite cell nucleus, probably co-transported with BMP, since cell treatment with dorsomorphin was able to inhibit RGMa nuclear localization. Additionally, we found that RGMa can induce satellite cell proliferation and also their commitment with differentiation upon provided and favorable conditions. Our results provide new information about the genetic heterogeneity and behavior of satellite cells cultivated *in vitro* in response to RGMa.

INTRODUCTION

Skeletal muscle stem cells, the satellite cells, accommodates a cellular source for muscle growth and regeneration. Satellite cells originate from positive Pax3 and Pax7 precursors that are present in the somite dermomyotome and remain quiescent in the adult musculature under physiological conditions (Maroto et al., 1997; Relaix et al., 2004, 2005; Seale et al., 2000). In response to diverse growth and repair stimuli, the satellite cells are activated, proliferate, self-renew and differentiate in mature muscle fiber (Yin et al., 2013; Kuang & Rudnicki, 2008; Montarras et al., 2005).

These cells are intimately associated with myofiber sarcolemma, beneath the basal lamina and Alexandre Mauro (1961) first named them satellite cells due to their distinct anatomical deployment. They can also be identified by the expression of several markers, specially by Pax7, the main defining factor for this cell type (Seale et al., 2000). Apart from their localization, morphological analysis and marker expressions,

satellite cells comprise a heterogeneous population of precursor cells (Oustanina et al., 2004): each round of cell division can give rise to two identical daughter cell (symmetric division) or generate one satellite stem cell and one myogenic progenitor cell (asymmetric division) committed to the myogenesis (Collins et al., 2019; Dumont et al., 2015; Yin et al., 2013). Although there are well known myogenic markers, the expression pattern can be variable between the cell populations, even though all of them maintain their myogenic potential.

Satellite cells are settled in a niche with high diversity of components, including myofiber, the basal lamina, different types of interstitial cells and vascular and neural networks. A complex dynamic network provides structural support, mechanical and chemical signals to regulate stem cell quiescence, self-renew and activation (Rezza et al., 2014). These components are indispensable to ensure the satellite cells functions and muscle regenerative capacity (Yin et al., 2013; Bentzinger et al., 2013).

Growth factors were already identified operating functions in satellite cells. Receptors and ligands from TGFβ superfamily control the cellular growth, progenitor activation, fibrosis and generation of adipose and ectopic bone tissue (Glass et al., 2010; Serrano et al., 2011; Sartori et al., 2013). Bone morphogenetic protein (BMP) are a subclass of the TGFβ family initially described as a potent inhibitor of muscular differentiation (Amthor et al., 1998; Rantapaa-Dahlqvist et al., 2003; Frank et al., 2006), although low levels of BMP is able to induce the Pax3 expression during primary myogenesis (Amthor et al., 1998; 1999). Recent works have been showing positive BMP effects during the satellite cell proliferation, fetal muscle progenitors (Wang et al., 2010) and gain of muscular mass (Sandri et al., 2013). A mouse model with decreased nuclear localization of BMP2 led to muscular, neurological and immune phenotypes, all of which are consistent with aberrant intracellular Ca^{2+} response (Felin et al., 2010; Freitas et al., 2019).

Repulsive Guidance Molecule a (RGMa) is a member of RGM family, first described during the growth and migration of axon in the neural tissue (Monnier et al, 2002). RGMa has been associated with neuron survival and differentiation mechanisms (Matsunaga & Chédotal, 2004; Matsunaga et al., 2006; Koeberle et al., 2010). Blocking the RGMa action with antibody treatment in areas of spinal cord damages has been shown to be effective in inducing neuronal regeneration (Hata et al., 2006; Doya et al., 2006).

Recent functional studies have revealed new functions for RGMs in non-neural tissues, including the origin, proliferation and differentiation of skeletal muscle cells

(Jorge et al., 2012). *In vitro* studies showed that RGMa can induce an increase in cell size and in the number of nuclei in mature myotubes, suggesting its involvement in skeletal muscle cell hypertrophy and hyperplasia (Do Carmo Costa et al., 2021; Copola et al., 2020; Martins et al., 2015).

The present work investigated RGMa expression pattern and possible biological functions during proliferation and differentiation stages of satellite cells isolated from mice in order to characterize the molecular mechanisms induced by this axon guidance molecule during the regeneration of skeletal muscle tissue.

MATERIALS AND METHODS

Animals

Wild-type C57BL/6J male mice from 6 to 8 week old were obtained from CEBIO according to CEUA/UFMG approved protocols. Animals were maintained on standard conditions at a constant temperature of 24°C under an artificial 12h light and 12h dark cycle with *ad libitum* access to water and food.

Satellite cell isolation and culture

Single myofibers were isolated from extensor digitorum longus (EDL) muscle from three C57BL/6J male mice as previously described (Rosenblatt et al., 1995; Wada et al., 2002; Pasut et al., 2013). Briefly, tendon-to-tendon EDL muscles were digested with 0.2% collagenase type I (Gibco) in DMEM high glucose (Dulbecco's modified Eagle's medium-high glucose and L-glutamine; Gibco), for 90 min at 37°C, under shaking. The digested muscle was then transferred to a new plate containing warm growth medium (GM: DMEM high glucose, supplemented with 20% Fetal Bovine Serum (FBS, Gibco), 1% chick embryo extract (CEE, US Biological Life Science) and 1% antibiotic-antimycotic (Gibco). Individual myofibers were released by repeatedly trituration using a plastic pipette coated with Horse Serum (HS). Live single myofibers were transferred to a new pre-warmed dish coated with HS.

Myofibers were maintained in GM at 37°C and 5% CO^2. After 4 days of incubation, myofibers were removed from the well. GM was replaced every 3 days. When reached a sufficient number to run the experiments, activated satellite cells were trypsinized and seeded at the density of $2x10^3$ cells/well in a 96-well culture plates.

Primary myoblasts isolation

Primary myoblast culture was prepared from hind limbs of five C57BL/6J neonates up to 3 days of birth. Muscle were dissected, minced and dissociated by a 60 min incubation in 0.2% collagenase type I (Gibco) at 37°C. After centrifugation at 300 x g cells were incubated again in a 2,5% trypsin solution for 30 minutes at 37°C. After centrifugation cell were cultured in DMEM high glucose (Gibco) supplemented with 20% FBS (Gibco) and 1% antibiotic-antimycotic (Gibco) and incubated for 1h in a humidified 5% CO^2 incubator at 37 °C to allow fibroblast adherence. Myoblast enriched culture were plated on Matrigel (BD Bioscience) coated dishes to promote the preferential attachment and growth of myoblasts.

Immunofluorescence staining in myofibers, isolated satellite cells and primary myoblasts

Immunofluorescence staining was performed in the following conditions: (i) myofibers right after their isolation; (ii) myofibers after 24h of incubation; activated satellite cells after (iii) four and (iv) 8 days in culture and (v) in primary myoblasts isolated from neonatal mice muscle. Briefly, cells were fixed with 4% PFA and incubated with 0.1M glycine to block aldehydes. Cells were then permeabilized with 5% goat serum and 0.2% Triton X-100 in PBS, for 30 min, followed by the incubation with anti-RGMa (Abcam, diluted 1:200 in PBS), anti-BMP4 (Santa Cruz, diluted 1:200 in PBS) or anti-Smad1/5/8 (Santa Cruz, diluted 1:200 in PBS) antibodies at 4°C overnight. After washing, samples were incubated with secondary antibody (Alexa Fluor 488, Molecular Probes, diluted 1:500 in PBS), for 1 h at room temperature. Nuclei were counterstained with DAPI (Invitrogen). Immunofluorescent images were captured using an Olympus Q-Color3 imaging system with an Olympus BX50 fluorescence microscope and Image Pro Express software (version 4.5).

RGMa subcellular localization prediction

PSORT II program https://psort.hgc.jp/psort/helpwww2.html#nncn was used to predict RGMa possible subcellular localization based on its signal peptide amino acid sequence.

Dorsomorphin treatment

Small molecule dorsomorphin (Sigma-Aldrich) was used to block BMP signalling in satellite cells. After 6 days of culture, activated satellite cells were treated with 5 μM

of dorsomorphin. Cells treated with DMSO, the vehicle of dorsomorphin, were used as control. Cells were incubated for an additional 2 days at 37 ºC and 5% CO^2 and the effects were assessed by immunofluorescence, as described above.

RGMa treatment

After 24h seeding, activated satellite cells were treated with recombinant RGMa protein (50 ng/ml; R&D Systems) in GM or differentiation medium (DM: DMEM supplemented with 2% horse serum and 1% antibiotic-antimycotic), for 2 and 5 days at 37 ºC and 5% CO^2. GM and DM were replaced every 3 days.

Cell viability and proliferation assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Life Technologies] assay was used to detect cell viability and proliferation after each treatment, according to manufacturer's protocol. Briefly, MTT was diluted in culture medium (MTT 1: 9 Medium), added to each well and then incubated for 2 h at 37ºC and 5% CO^2. The MTT solution was carefully removed, and 200 µl of isopropanol acid (100 ml isopropanol: 134 µl hydrochloric acid) was added to each well. The solution was then transferred to a 96-well plate (50µl/well) and the absorbance was measured at 595 nm using the spectrophotometer ELX800, BioTek®. All experiments were performed in triplicate.

RT-qPCR

Total RNA was isolated from primary satellite cells using Tri-Reagent (Sigma-Aldrich), following manufacturer's instructions. RNA integrity was monitored by RNA electrophoresis followed by the conversion of 1 µg of total RNA in cDNA using High-capacity cDNA reverse transcription kit instructions (Thermo Fisher). All qPCR reactions were performed using the Rotor-Gene RT-PCR system (Qiagen). Each reaction contained 1x iTaq Universal SYBR Green Supermix (Bio-Rad) and 0.4–0.6 µM of each primer and 1 µl of cDNA (diluted 1:10), in a final volume of 10 µl. Samples were run in triplicates using the following thermal cycle conditions: 95°C for 2 min, followed by 45 cycles of a three-step reaction, 94°C for 15 sec, 60–62°C for 15 sec and 72°C for 20 sec; and by a final cycle of 72°C for 5 min. The dissociation step was performed at the end of the amplification step to allow the identification of the specific melting temperature for each primer set. Gene expression fold change was calculated

using the REST 2009 software (Pfaffl 2001; Pfaffl et al. 2002), normalized against the expression of gapdh.

Primers used in this work are as follow: GAPDH (AGGTCGGTGTGAACGGATTTG and TGTAGACCATGTAGTTGAGGTCA), CD34 (TGCTTACACATCATCTTCTGCTC and CAGCCTCCTCCTTTTCACAC), PCNA (ACATTGGAGATGCTGTTGTG and CAGTGGAGTGGCTTTTGTG), Myf5 (ACTGGCGTGTCTCCTCTCT and TCAAACTGGTCCCCAAACTC), MyoD (GTGGCAGCGAGCACTACA and GACACAGCCGCACTCTTC), MyoG (TGAGAGAGAAGGGGGAGGAG and CGGTATCATCAGCACAGGAG), Ki67 (GCAGCCTCTTCACCCAAA and GGCACTTTCACCTTCATCCA), Ski (CGACTGAATCTGCCACTGTC and AGAGCGGAGGGCTTTTGTAT) Mef2d (CCTGTTTTCTTTCCCACCTG and ATGGAGAAGGTGGTGTGAGG) and RGMa (ATGCAGCCGGGGAGCAGGCG and CCCAAGGCTCAGGGCAGTCG).

Statistical analysis

Statistical analysis was performed using the Prisma software 7.0 version. MTT data was analysed using the two-tailed Student's t-test to compare differences between two groups, while one-way ANOVA was performed for all experiments that require a simultaneously comparison between three or more groups within one categorical variable followed by a Tukey's post hoc test. All the experiments were performed at least three times. Results were considered significant at $p<0.05$ (*), $p<0.01$ (**) and $p<0,001$ (***). Data are presented as mean±SD.

RESULTS

Standardization of satellite cells isolation

EDL (extensor digitorum longus) muscle was collected from tendon to tendon and submitted to an enzymatic dissociation step with 0.2% collagenase type I. Thus, we observed slightly disaggregated myofibers (Figure 1A). To allow the complete dissociation of the myofibers, we resuspended the sample with the aid of a pasteur pipette, promoting the mechanical dissociation and complete detachment of the myofibers (Figure 1B). We collected the bright, long and intact fibers (Figure 1C) and plated them in rich growth medium to allow the newly activated satellite cells to migrate from the periphery of the fibers to the culture dish from the 4th day onwards (Figure

1D). From the 8th day, the satellite cells are able to spread across the plate and we replate them when they reach a satisfactory confluence (80~90%) (Figure 1E-F).

Immunostaining revealed the expression of RGMa in satellite cells nuclei

We investigated if RGMa was expressed in quiescent and activated satellite cells, using immunofluorescence. For this, isolated myofibers were obtained from mice EDL muscle and cultured up to 8 days. Immunostaining in quiescent cells was performed in myofibers right after the harvesting process (0h) and in activated satellite cells was assessed after 24h (1d), 4 and 8 days of culture (Figure 2).

Our results revealed the expression of RGMa in particular myonuclei at 0h (Figure 2A-C), followed by its expression in all myonuclei after 24h of culture (Figure 2D-F). Curiously, after 4 and 8 days of culture, RGMa expression in activated satellite cells was restricted to the nuclei (Figure 2G-I).

We have also determined the expression pattern of key myogenic markers during satellite cells culturing for up to 10 days, by RT-qPCR (Figure 2M-O). Our results showed that up to five days of culture, only MyoD and Pax7 were found to be upregulated (Figure 2M). Gene expression has changed in these cells only after 10 days of culture, when we could observe the upregulation of Pax7 (marker of satellite cell), Myogenin (a marker of myogenic differentiation) and RGMa (Figure 2O).

We have also obtained the expression pattern of RGMa in cells isolated from the digestion of the whole muscle cut in small fragments, resulting in a culture containing both satellite cells and myoblasts (Figure 3). In this case, RGMa could still be found in the nuclei and also in the cytoplasm of the isolated cells (Figure 3).

All together the expression pattern analysis suggested that: (i) RGMa is expressed in activated satellite cells and (ii) its subcellular localization depends on the differentiation stage of the cell: nuclear while activated stem cell and nuclei and cytoplasmic while myogenic determined cell.

RGMa might be transported to the nuclei by other proteins

RGMa is known as GPI-anchored protein, which means that it is expected to be found in the plasmatic membrane of the cells (Monnier et al., 2002). In this work, we could clearly find RGMa in the nuclei of activated satellite cells. So, we decided to investigate if RGMa amino acid sequence could provide us with clues of other RGMa

subcellular localization besides the membrane. For this, we have used the pSORTII software.

We found that RGMa present a cytoplasmic prediction (55.5%, Table 1). No predictable nuclear localization site (NLS) could be found in the RGMa amino acid sequence (Table 1). Additionally, we could find two dileucine motifs in the RGMa amino acid sequence, one located at the N-terminus (LL75) and the other at the C-terminus of the protein (LL419, Table 1), which are motifs present in multiple membrane-bound proteins that have been selected as cargo to be transported to the endosomes and lysosomes by clathrin-coated vesicles (Bonifacino & Traub, 2003).

Table 1: Result from PSORT II program analysis for Bmp2, RGMa and Neogenin

	Bmp2 (*)	**Rgma**	**Neogenin Isoform 1**	**Neogenin Isoform 2**
# acess	NP_031579.2	NP_808408.2	NP_032710.2	NP_001036217.1
NLS	Pat7: PELGRKK at 65 Pat7: PLHKREK at 312 Bipartite: KREKRQAKHKQRKRLKS at 315	none	Pat4: KKKR at 1195	Pat4: KKKR at 1168
Prediction reliability	Nuclear 76.7	Cytoplasmic 55.5	Nuclear 89	Nuclear 89
Duleucine motif in the tail	LL at 50 LL at 51 LL at 56 LL at 95 LL at 228	LL at 75 LL at 419	none	none

(*) Felin et al, 2010

Therefore, RGMa nuclei localization could not be predicted by its amino acid sequence, which suggests that RGMa might be co-transported to the nuclei by other proteins, which were not investigated thus far.

Immunostaining in myofibers revealed the expression of RGMa, BMP4 and Smad1/5/8 in myonuclei

Isolated myofibers were obtained from mice EDL muscle, as previously described, and cultured in rich culture mediuim up to 48h. Immunostaining was performed to investigate the localization of RGMa, BMP4 and Smad 1/5/8 expression in the myofiber.

All three markers showed their expression in the nuclei present in the myofiber (Figure 4). This result suggests that the RGMa-BMP signaling pathway is co-localized in the activated satellite cell nuclei.

Inhibition of BMP pathway by dorsomorphin decreases RGMa immunostaining in satellite cells nuclei

RGMa is a regulator of the BMP signaling pathway and its N-terminal domain consists in a high-affinity interaction site for BMP2 ligands (Healey et al., 2015). BMP2 can produce a particular isoform that present three NLS motifs in its sequence, which directs this protein to the cell nuclei right after translation (Felin et al., 2010). For this reason, we decided to evaluate if RGMa nuclei staining in activated satellite cells could be detected in cells treated with dorsomorphin, a small molecule known to block BMP signaling. For this, activated satellite cells were cultivated for four days and then treated with 5 μM of dorsomorphin (DM) or DMSO, the DM vehicle, as a control. RGMa immunostaining was performed after two days of culture treatment.

Our results showed a clear decrease of RGMa expression in the nuclei of these cells (Figure 5A-C), compared with the control (Figure 5D-F).

This result suggests that the presence of RGMa in the nuclei of activated satellite cells is dependent of the BMP signaling pathway.

RGMa induces satellite cell viability/proliferation in growth medium

We next investigated RGMa possible biological functions in activated satellite cells.

We first considered the effects of satellite cells incubation with a mouse RGMa recombinant protein, on cell viability/proliferation. Viability index was obtained after two and five days of treatment, using the MTT assay.

Our results show that satellite cells treatment with RGMa recombinant protein induces an increase in cell viability index, both after 2 (Figure 6B) and 5 days (Figure 6C) of incubation, compared to the control that received only growth medium.

To confirm the induction of cell viability/proliferation by RGMa, we compare the effects of RGMa and LIF in satellite cell culture, using MTT assay. Leukemia inhibitory factor (LIF) belongs to the interleukin-6 superfamily and is known to act regulating cell proliferation, differentiation, survival, and also maintaining the pluripotency state and self-renewal of muscle stem cells (Jiang et al., 2002; Metcalf, 2003; Broholm et al., 2012; Ito et al., 2016, Santos et al., 2020).

Our results show that satellite cells treatment with RGMa recombinant protein induced a greater increase in cell viability index when compared to the one achieved by LIF treatment (10ug/ml), both after 2 (Figure 8B) and 5 days (Figure 8C) of incubation.

RGMa induces no effects in satellite cell viability/proliferation in differentiation medium

We have also investigated if RGMa would be able to induce satellite cell viability/proliferation in differentiation medium. Viability index was again obtained after two and five days of treatment, using the MTT assay.

Our results showed that RGMa was not able to induce any effect on cell viability/proliferation while these cells were cultivated during two and five days in differentiation medium (Figure 7B-C). When the effects of RGMa and LIF, were compared in differentiation medium, we conclude that neither LIF was able to cause any response on satellite cell in this environment (Figure 8D-E).

RGMa induces effects on the expression of myogenic markers leading to cell proliferation and commitment to myogenic development

We have also assessed the effects of satellite cell treatment with RGMa during two and five days, in growth and differentiation medium, by RT-qPCR.

In growth conditions, RGMa could induce the upregulation of Myf5 and MyoD expressions after two days of treatment, compared to the control (Figure 9A). RGMa could also downregulate the expression of the cell cycle markers, Ki67 and Ski (Figure 9A). After five days of treatment, RGMa could induce the upregulation of the satellite cell surface marker CD34, of the cell cycle marker Ki67 and of the myogenic transcription factors Myf5 and Mef2d (Figure 9B).

In differentiation conditions, RGMa treatment for two days induced the downregulation of Myf5 expression and the upregulation of Desmin, compared to the control (Figure 9C). After five days of culture, however, RGMa induced the downregulation of MyoD expression, and the upregulation of Myogenin, RGMa, Ki67 and Desmin, compared to the control (Figure 9D).

All together these results suggest that RGMa can induce the expression of the Ki67 proliferation marker both in proliferative and differentiation conditions. RGMa could also induce the expression of early myogenic markers during proliferation conditions while inducing the expression of differentiation markers when cells are cultivated in differentiation medium.

DISCUSSION

RGMa is an axon guidance molecule originally found working as a repulsive clue to the growing axons. New evidences have been showing that RGMa has functions in different cell types, including in skeletal muscle. RGMa was found to be expressed in pioneer muscular cells in the dermomyotome of chicken somites, colocalized to the Pax7+ cells, a marker for satellite cell precursor (Jorge et al., 2012). RGMa biological functions were investigated in C2C12 and primary skeletal muscle cells and suggested that this axon guidance molecule could induce cell hypertrophy and hyperplasia (Martins et al., 2015). In this work, we have investigated the biological effects and the mechanisms induced by RGMa in skeletal muscle stem cells, the satellite cells.

We first determined the expression pattern of RGMa in these cells. Two different protocols to isolate satellite cells were used in this work: (i) the fiber isolation and (ii) the primary myoblasts isolation. RGMa was found to be expressed in particular myonuclei of the myofibers at the moment of isolation, but we were not able to confirm that these nuclei were in fact from satellite cells. A double-staining with anti-Pax7 antibody should be performed to confirm that. After 24h of culturing, RGMa expression could be detected in all myonuclei of the myofiber, suggesting that this axon guidance molecule is expressed in activated satellite cells. After activation, these cells migrate from the myofiber to colonize the well with new myogenic precursors (Siegel et al., 2009; Danoviz & Yablonka-Reuveni, 2012). We have cultivated these cells up to 10 days and we could detect the presence of RGMa in the nuclei of these cells up to 8 days of culture. Only at 10 days of culture that we could detect the expression of myogenic differentiation markers, meaning that these cells produced from the activation of quiescent satellite cells can keep their stem cell expression pattern during culturing. However, RGMa is a known GPI-anchored protein, which means it is supposed to be found in the plasmatic membrane. How can RGMa reach the satellite cell nuclei and what is it doing there?

The nuclear portion of the cell is responsible to regulate numerous cellular events such as cell cycle, transcription and gene regulation. The transport of proteins and RNA across the nuclear envelope is a complex process that requires a variety of molecules (Hoelz et al., 2011; Knockenhauer & Schwartz, 2016). Small molecules from 40-45 KDa can diffuse easily between cytoplasm and nucleus, but as protein size increase, its ability to transpose diminishes rapidly (Mohr et al., 2009; Schmidt et al., 2016). Large molecules usually present a nuclear localization signal (NLS) to enter the nucleus (Jans & Huebner, 1996), but nearly 50% of the human nuclear proteome does not have a predictable NLS (Tessier et al., 2020). Proteins that do not present

de NLS sequence can be translocated to the nucleus by binding to a co-transporter that contains a nuclear localization signal (Lesage et al., 2004; Jerome & Paudel, 2014). We have performed the analysis using PSORT II software and found that RGMa does not exhibit NLS in its amino acid sequence, which suggests that RGMa is not able to address itself to the nucleus. RGMa might need another protein to complete its co-transport.

RGMa was described to play its canonical effects through the neogenin receptor (Rajagopalan et al., 2004, De Vries & Cooper 2008; Harada et al., 2016; Fujita & Yamashita, 2017) and its non-cannonical effects are driven by the bone morphogenetic protein (BMP) (Babitt et al., 2005; Samad et al., 2005; Mueller, 2015; Siebold et al., 2017). Curiously, Felin and colleagues (2010) identified a nuclear variant of BMP2 presenting three NLS motifs in its amino acid sequence. As the N-terminal domain of the RGMa protein can interact with BMP2 (Tian & Liu, 2013; Healey et al., 2015), this result suggested us that RGMa could be being co-transported into the cell nucleus via interaction with BMP. In 2015, Mueller suggested a cluster formation between BMP-RGM-Neogenin that must enhance the modulation of involved molecules and he left opened an unanswered question about which mechanism, other than endocytosis, could explain the possibility of membrane-anchored RGM protein improving BMP and/or Neogenin signalling. Moreover, the dileucine motif, also present in RGMa tail, was identified as a clathrin-dependent sorting signal by Letourneur and Klausner in 1992. The dileucine motif is able to bind to part of the AP heterodimer, an assembly polypeptide complex, part of the clathrin vesicle formation (Miller et al., 2007 and 2011), corroborating to the data showed by Siebold and colleagues that the interaction of RGM and BMP might undergo endocytosis.

In order to verify if RGMa could be linked and co-transported by BMP into the nucleus, we blocked the BMP signalling in satellite cells with dorsomorphin (Yu et al, 2008; Anderson et al, 2008; Sanvitale et al, 2013) and found a very weak staining of RGMa in the nuclei, compared to the control. The mechanism involved in the nuclear transport of RGMa by BMP was not cleared yet.

We have also investigated the effects of RGMa in satellite cells cultivated in growth and differentiation medium. RGMa was capable to increase the cell viability after two and five days in growth medium, even more efficiently than LIF, a known stem cell proliferative factor. No effects on cell viability were observed when these cells were cultivated in differentiation medium.

Since their identification as a residing dormant cell beneath the basal lamina of mature skeletal muscle fibers (Mauro et al., 1961), the satellite cell has been an attractive aspirant for the adult skeletal muscle stem cell. Activated satellite cell enter in the cell cycle and each round of cell division gives rise to two daughter cells with similar or divergent fates (Kuang et al., 2007; Kawabe et al., 2012; Le Grand et al., 2009; Liu at el., 2012). The symmetric division contribute to the maintenance of satellite steam cell poll, while the asymmetric division generate one satellite stem cell and one myogenic progenitor cell, expressing MyoD and Myf5 (Collins et al., 2006; Kuang et al., 2007; Sacco et al., 2008; Liu et al., 2012).

Gene expression analysis of satellite cells cultivated in growth medium and treated with RGMa revealed, once again, their heterogeneity. After two days of treatment, RGMa induced the upregulation of Myf5 and MyoD, which are transcription factors required for the determination and propagation of myoblasts (Rudnick et al., 1993). Both MyoD and Myf5 are expressed in activated satellite cells, but Myf5 expression is heterogeneous in MyoD+ activated cells (Cornelison & Wold, 1997; Cooper et al., 1999). The time course studies revealed that MyoD and Myf5 did not always coincide with each other during myogenic progression *in vitro* and *in vivo* (Smith et al., 1994; Kitzmann et al., 1998). Asymmetric cell division gives rise to transcriptionally divergent satellite cells. Pax7+/Myf5- satellite cells can give rise to both Pax7+/Myf5+ and Pax7+/Myf5- cells. Pax7+/Myf5+ daughter cells were majority in the apical side and more responsive to differentiation, whereas Pax7+/Myf5- daughter cells, basally positioned, were more susceptible to contribute to the satellite stem cell compartment (Kuang et al., 2007). MyoD can also be asymmetrically distributed into two daughter cells: Pax7+/MyoD+ and Pax7+/MyoD- cells (Liu et al., 2012).

Over the genes inhibited by RGMa treatment in growth medium, Ski and Ki67 had their expression downregulated. Ski is necessary during the terminal differentiation of skeletal muscle cells but not in the determination of cells to the myogenic lineage (Zhang & Stavnezer, 2009), so its inhibition was expected, since the cells seem to be inserted in the determination phase of myogenesis. Ki67 is a nuclear protein expressed in all proliferating vertebrate cells. *in vitro*, Ki67 expression levels are high in G2 phase and mitosis, configuring it as a late marker of cell-cycle entry (Endl & Gerdes, 2000). After cell cycle exit, low level of ki67 expression remained but inappreciable in fully quiescent differentiated or senescent cells (Sobecki et al, 2017). Given this scenario of gene expression, it can be suggested that RGMa acts in the proliferation of satellite cells.

After five days of culture, we found the upregulation of CD34, Myf5, Ki67 and Mef2d transcripts in satellite cells treated with RGMa. Beauchamp and colleagues (2000) proposed that CD34 and Myf5 are intimal related, since the Myf5 locus is active in all CD34+ quiescent satellite cell and all Myf5+ precursors express CD34. The CD34 marker does not act in stem cells but it is expressed by precursors that are committed to a specific fate and have become arrested and held in reserve to a subsequent activation.

Mef2d is part of Mef2 family of transcriptional regulators and has a critical role during myogenesis (Molkentin et al., 1995). Working closely to MyoD to accelerate skeletal muscle differentiation (Penn et al., 2004), Mef2d was shown to increase the proliferation rates through accelerating the G2-M phase transition (Ma et al., 2014). However, Mef2 proteins solely are not able to direct skeletal muscle differentiation in the absence of MRFs (myogenic regulatory factor) (Molkentin et al., 1995). Copola et al (2022) had found Mef2d as an upregulated transcript after 5 days of RGMa treatment in C2C12 cells. In satellite cells culture, during 5 days of RGMa treatment, the pool seems to divide into two groups: some of them overexpressing CD34 and Myf5, compromised with the muscular fate, and at the same time they are arrested and waiting for activation. Another part of the cells is proliferating, as they present an increase of Ki67 and are preparing to enter in the differentiation phase, when they overexpress Mef2d transcript.

After two days of culture in differentiation medium, we found that Myf5 was downregulated while Desmin was upregulated by RGMa treatment. Myf5 expression must occur during the determination stage, and in this case, we are one step forward from determination, with the cells preparing to enter in the differentiation. Desmin is a protein identified in the intermediate filaments of skeletal muscle (Geisler & Weber, 1982; Traub, 1985; Li et al., 1997). The first step in recognition of skeletal muscle myogenesis seems to be the beginning of Desmin synthesis in replicating myoblasts. The other muscle-specific proteins appear only in the post mitotic mononucleated myoblasts (Babai et al., 1990; Allen et al., 1991; Mayo et al., 1992).

After five days in differentiation medium, RGMa treatment downregulated the expression of MyoD, clearly demonstrating the deviation with determination fate and the entrance in a later stage, in which MyoG enhancing the expression of a subset of genes previously initiated by MyoD (Cao et al., 2006). MyoG appears to have a critical role in the terminal differentiation of the specified muscle cells (Hasty et al., 1993; Nabeshima et al., 1993), just like Desmin. The expression of Ki67 is significant, once

the satellite cells seems to exit the cell cycle, the point where it is possible to detect high levels of Ki67 transcription. Curiously, the RGMa transcript is also overexpressed. In previous work from our lab, RGMa was seen already inducing myogenic commitment at early stages of differentiation and cell fusion during the late ones (Copola et al., 2022).

CONCLUSIONS

RGMa is expressed in activated satellite cells nuclei and its presence in the nucleus might be BMP-dependent, considering RGMa disappearance after dorsomorphin treatment. Additionally, RGMa seems to induce satellite cells proliferation in the growth medium and differentiation in a poor serum content medium. The genetic background of satellite cells confirmed their heterogeneity and the role of the niche to manage their behaviour in different demands.

FIGURES AND LEGENDS

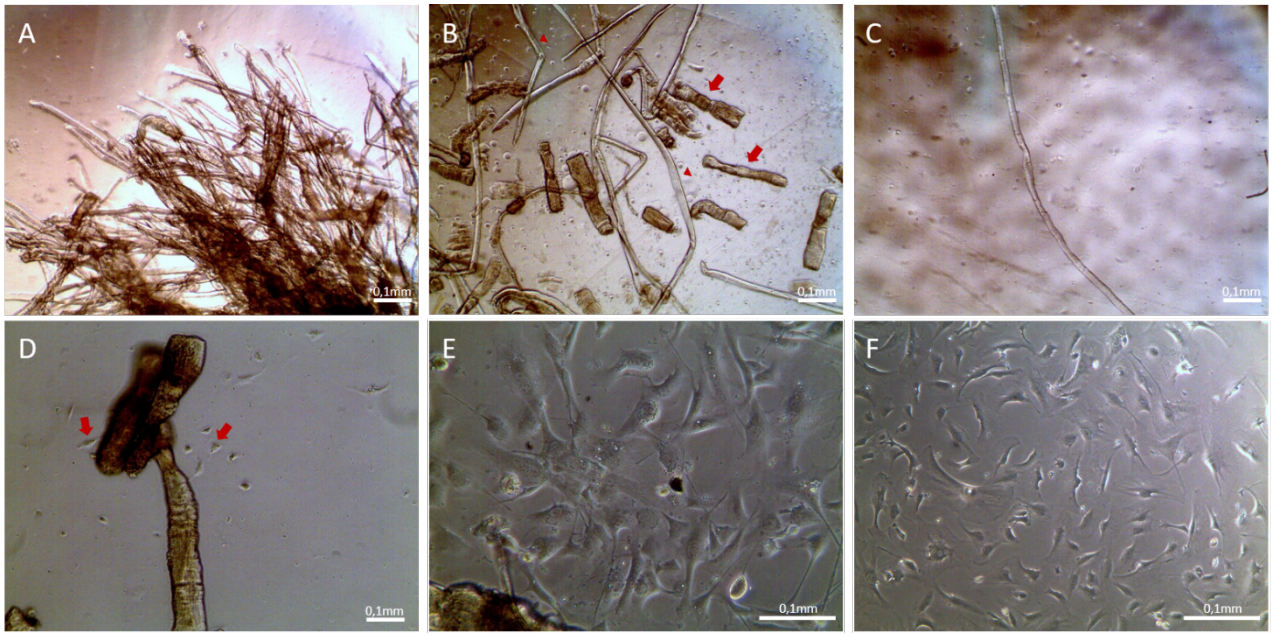

Figure 1. Isolation of satellite cells from skeletal muscle of adult mice.

(A) Myofibers after enzymatic dissociation and after mechanical dissociation (B), where it is possible to observe injured and super contracted myofibers (arrows) and intact myofibers (arrowhead). (C) Isolated intact myofibers to be plated. (D) Satellite cells adhered to the plate after 4 days of extraction (arrow) and maintained in rich growth medium. (E) Proliferating satellite cells after 8 days of extraction and after 10 days with sufficient confluency for replating. Images were taken with a 10x (A-D) and a 20x (E and F) objective.

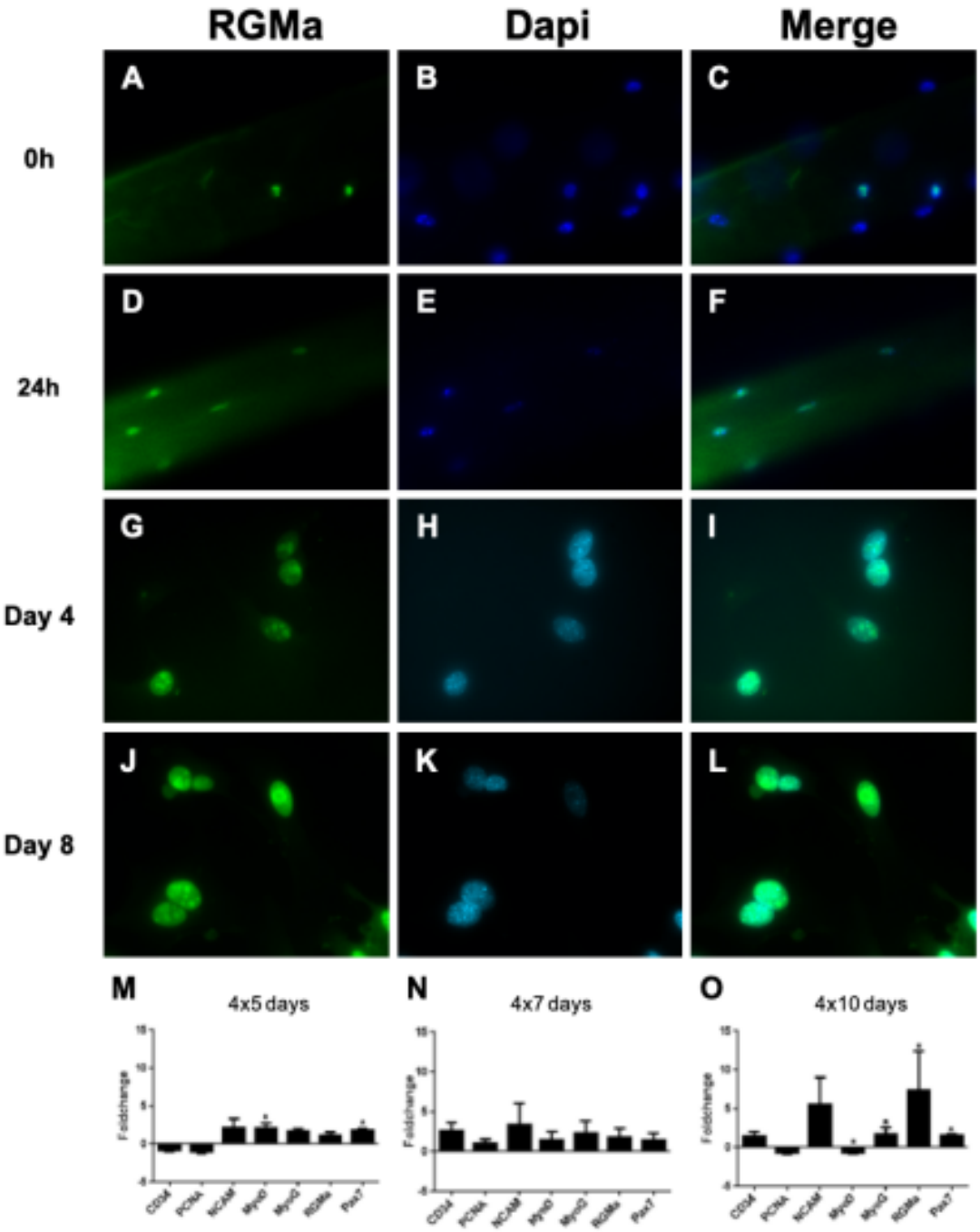

Figure 2. Satellite cells are expressing endogenous RGMa in the nuclei. Myofiber cultivated in suspension, during (A-C) and after 24h (D-F) of isolation procedure. Satellite cells cultivated at 4th (G-I) and 8th (J-L) days post myofiber plating into the culture dish. Representative images of immunofluorescence using anti-RGMa antibody (green). Nuclei were counterstained with DAPI (blue) and cells were examined by fluorescence microscopy. Gene expression pattern of satellite cells cultivated and harvasted on 4, 5, 7 and 10th days after myofiber seeded (M-O).

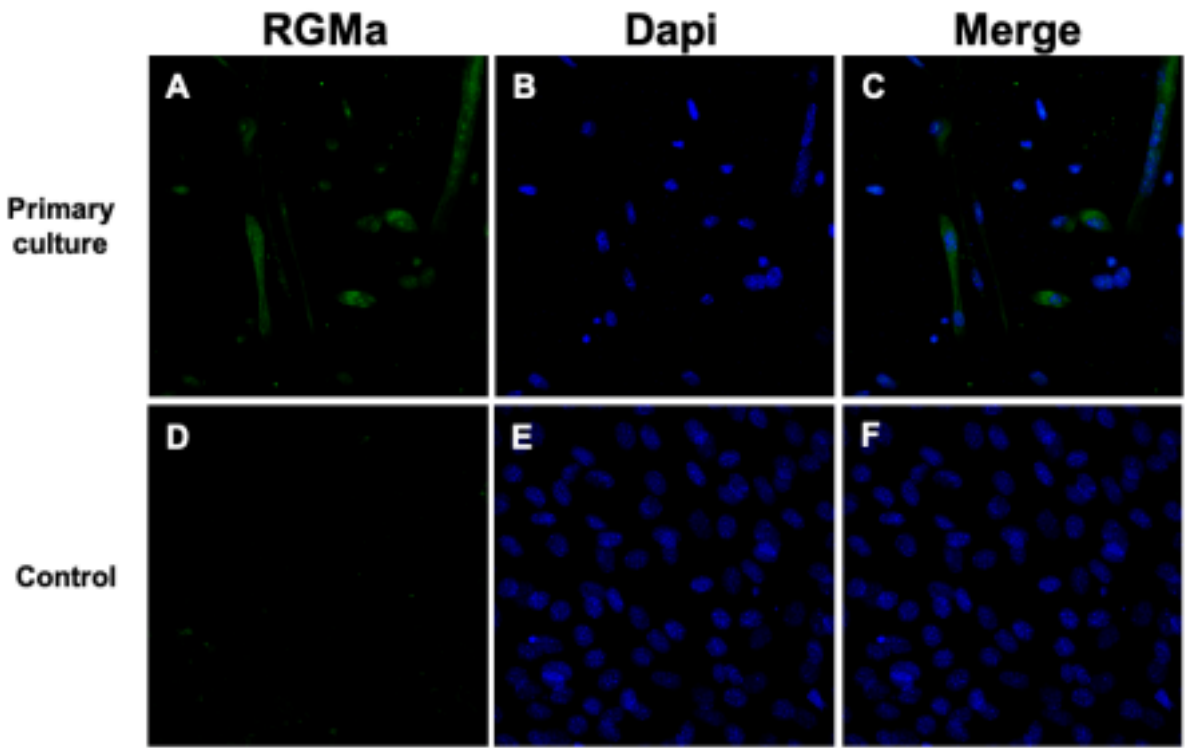

Figure 3. RGMa expression pattern in primary myoblast heterogeneous culture. Primary myoblast cells present RGMa staining in nucleus and in the cytoplasm. Representative images of immunofluorescence using anti-RGMa antibody (green). Nuclei were counterstained with DAPI (blue) and cells were examined by fluorescence microscopy.

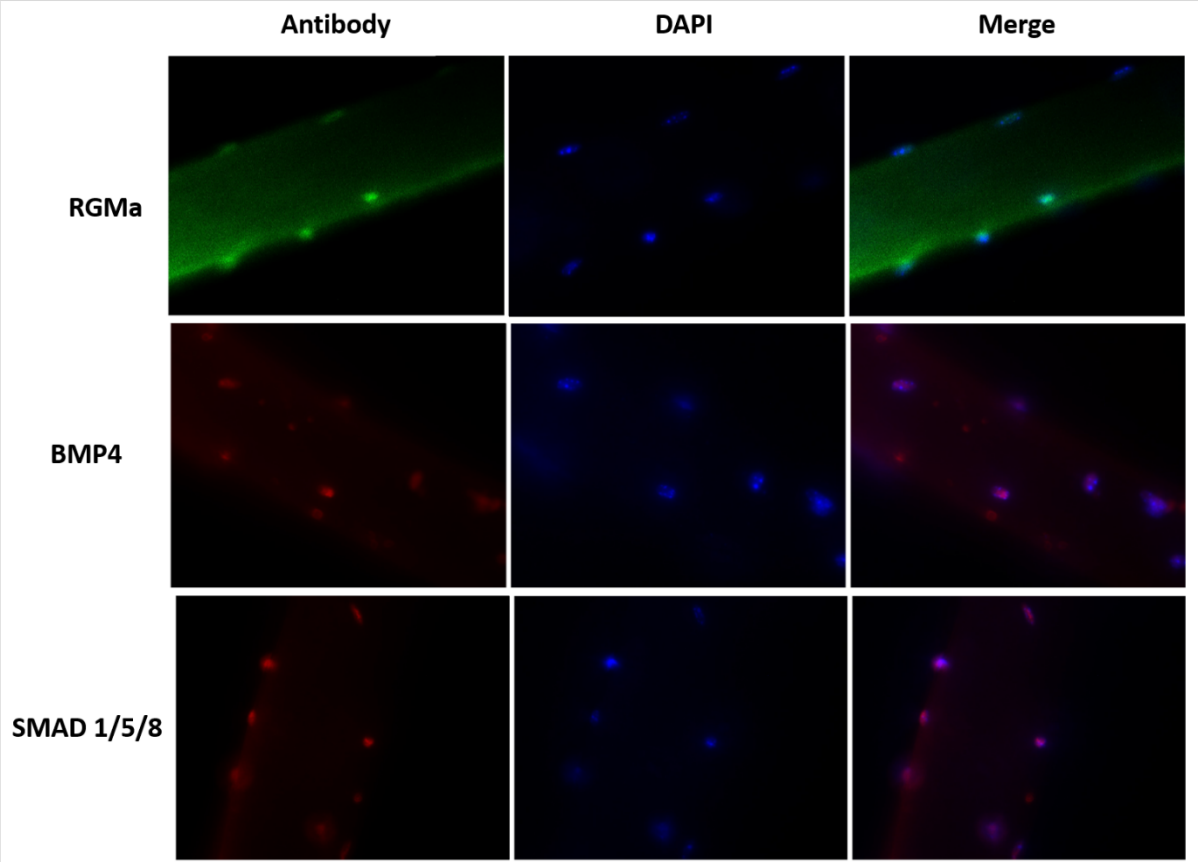

Figure 4. RGMa, BMP4 and Smad1/5/8 expression in myofibers. Primary myofibers 48h after isolation present RGMa, BMP4 and Smad1/5//8 staining in the nuclei. Representative images of immunofluorescence using anti-RGMa (green), anti-BMP4 and anti-Smad1/5/8 (red). Nuclei were counterstained with DAPI (blue) and cells were examined by fluorescence microscopy.

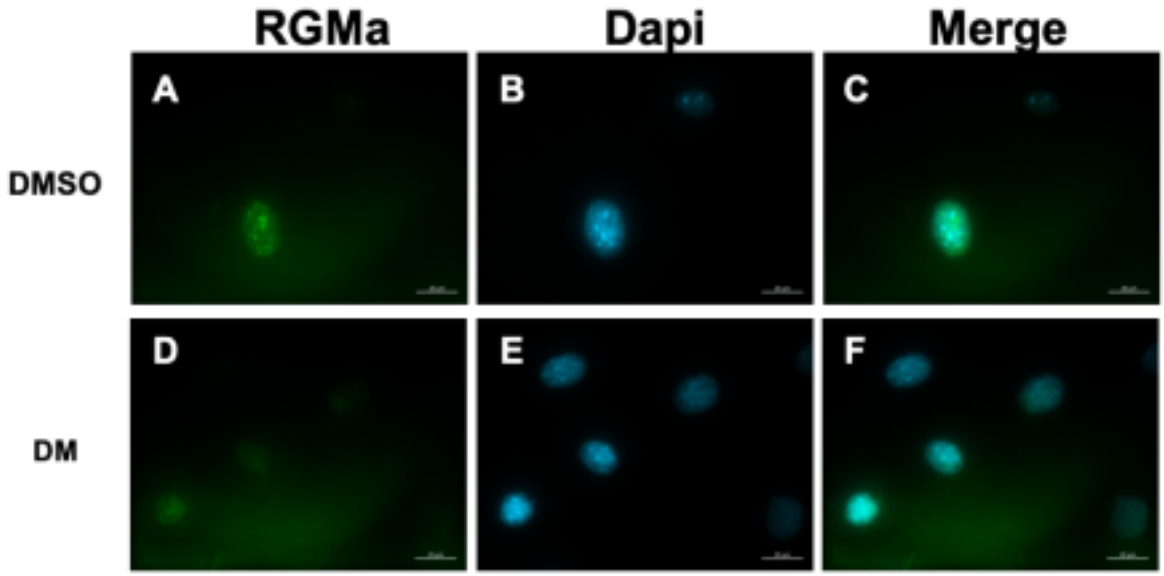

Figure 5. Satellite cells treated with dorsomorphin does not retain RGMa in the nuclei. Satellite cells cultivated until the 8th day post myofiber plating and treated with dorsomorphin (A-C) or DMSO (D-E) during 2 days. Representative images of immunofluorescence staining showing anti-RGMa antibody (green) and DAPI (blue).

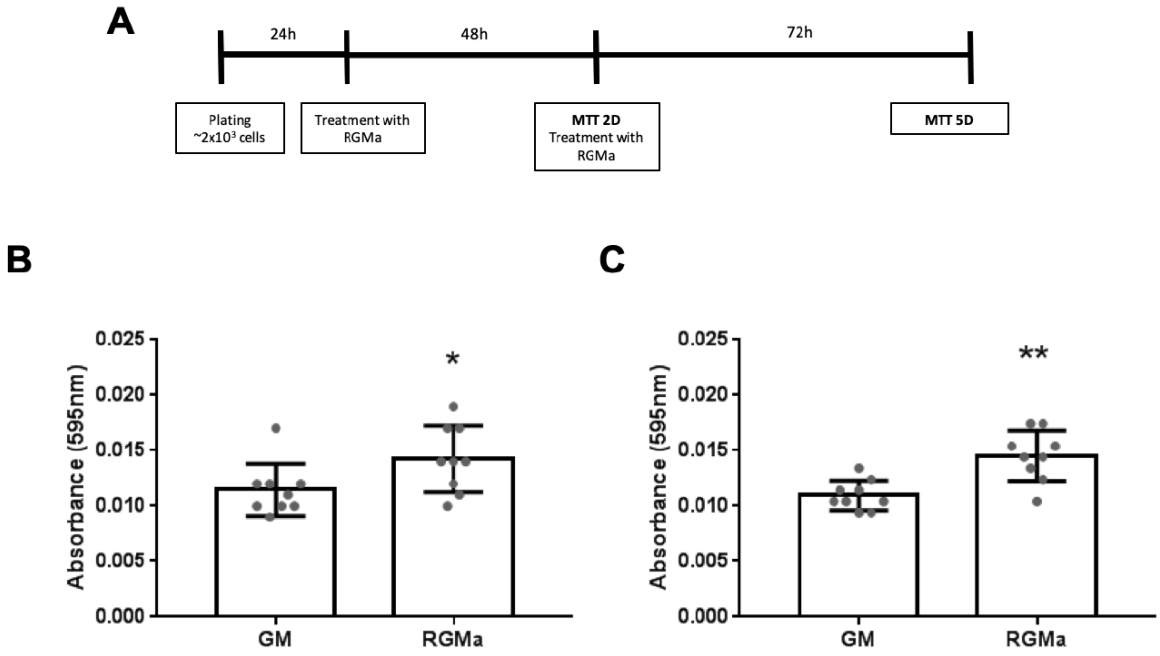

Figure 6. RGMa promotes cell viability in proliferative conditions. Experiment design (A). After 24h for cell adhesion, growth medium was replaced by new medium containing 50ng/ml of recombinant RGMa and cells were incubated for 2 days. On the second day, half of the wells were submitted to MTT assay, while the other half received another treatment of recombinant protein and was incubated for more 3 days, previously to MTT analysis. MTT shows RGMa effect on growth medium on day 2 (B) and on day 5 (C). Differences between groups were assessed for significance using unpaired student t-test. * p< 0.05 vs control and ** p< 0.01 vs control. Data are presented as mean± SD.

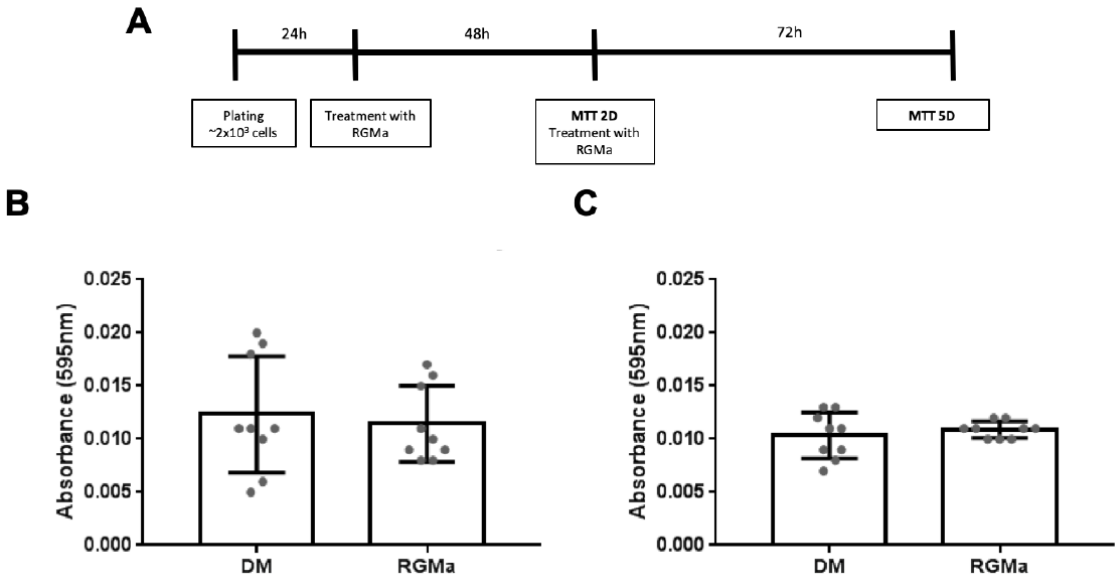

Figure 7. RGMa was not able to induce cell viability in differentiation conditions. Experiment design (A). After 24h for cell adhesion, growth medium was replaced by differentiation medium containing 50ng/ml of recombinant RGMa and cells were incubated for 2 days. On the second day, half of the wells were submitted to MTT reading, while the other half received another treatment of recombinant protein and was incubated for more 3 days, previously to MTT analysis. RGMa does not caused any effect in differentiation medium on day 2 (B) and neither on day 5 (C). Differences between groups were assessed for significance using unpaired student t-test. * p< 0.05 vs control and ** p< 0.01 vs control. Data are presented as

mean± SD.

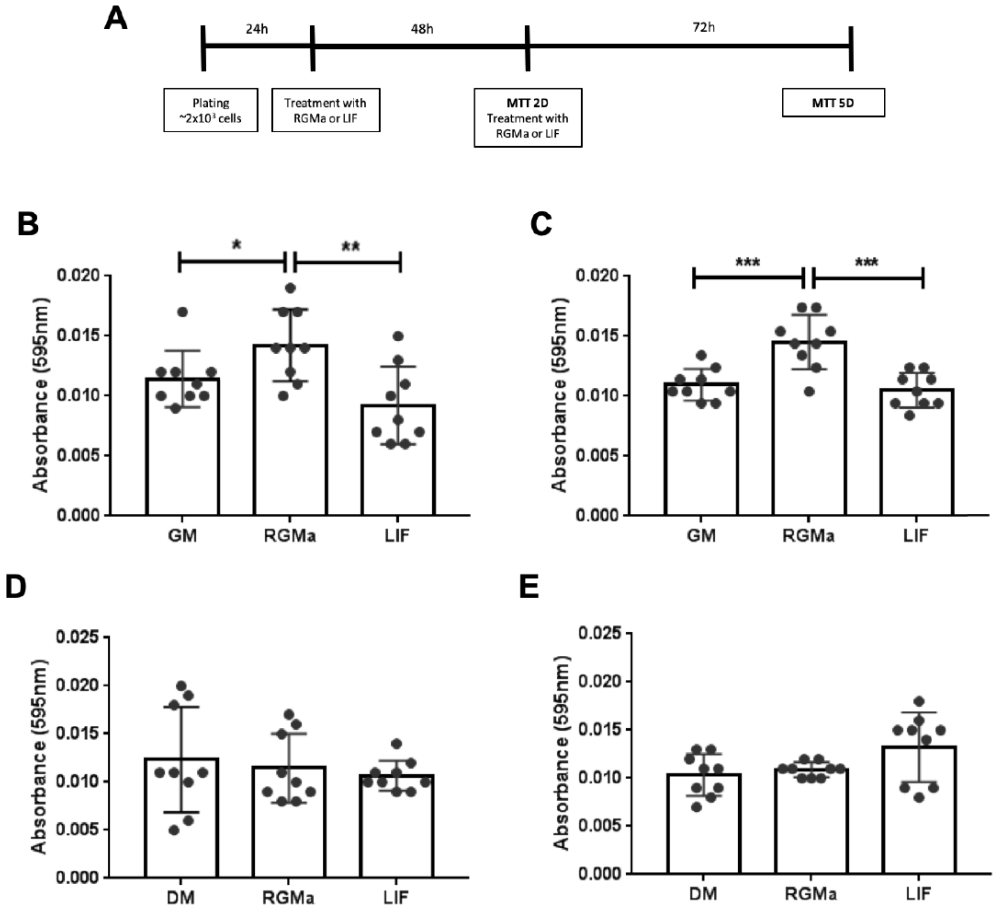

Figure 8. RGMa was more efficient to induce cell viability/proliferation than LIF. Experiment design (A). After 24h for cell adhesion, medium was replaced by new medium containing 50ng/ml of recombinant RGMa or 10ug/ml of LIF and cells were incubated for 2 days. On the second day, half of the wells were submitted to MTT assay, while the other half received another treatment and was incubated for more 3 days, previously to MTT analysis. Cell viability induced by RGMa effect on growth medium was greater than LIF effect both on 2 (B) and on 5 (C) days post treatment. On differentiation medium, nor RGMa neither LIF treatment was able to induce any effect on cell viability/proliferation on 2 (D) or 5 (E) days. Differences between groups were assessed for significance using one-way ANOVA test. ** $p < 0.01$ and *** $p < 0.001$. Data are presented as mean± SD.

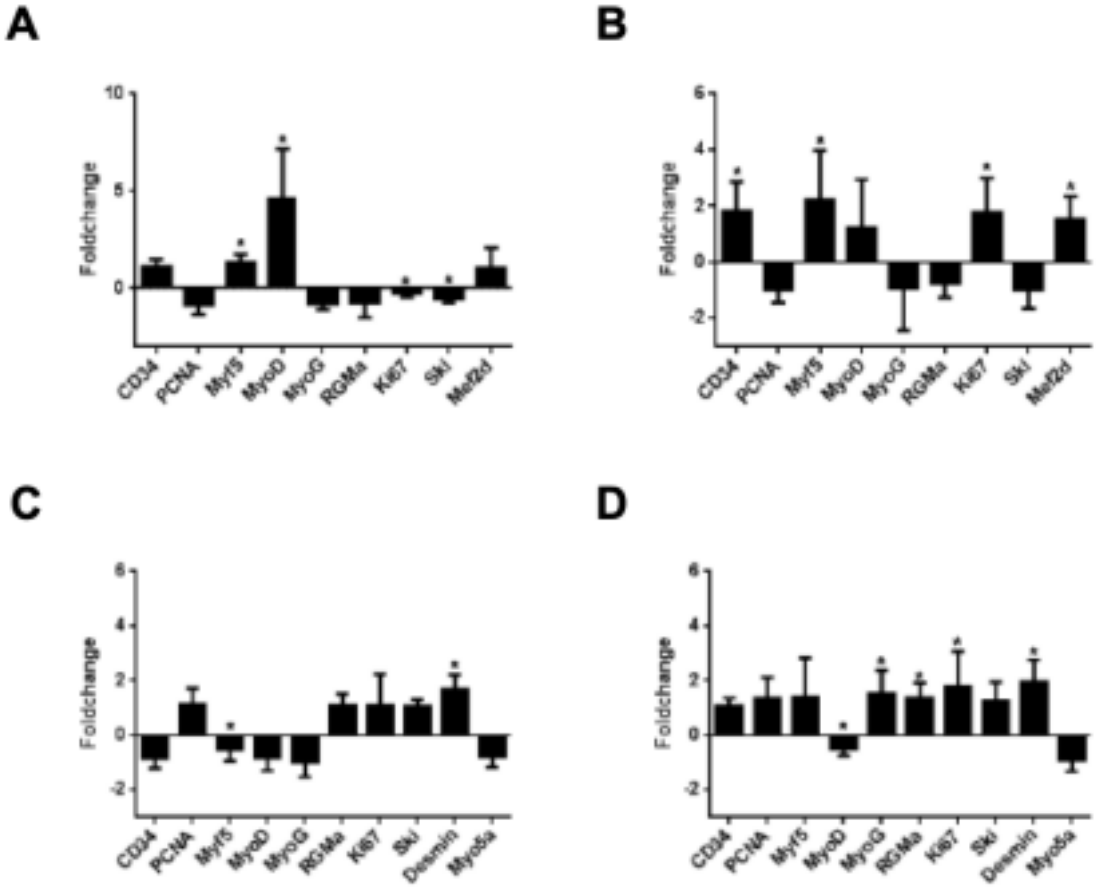

Figure 9. RGMa induces gene expression changes leading to cell proliferation and commitment to myogenic development. Analysis of gene expression in satellite cells treated with recombinant RGMa at 2 (A) and 5 (B) days in growth medium and in differentiation medium, also at 2 (C) and 5 (D) days of treatment. Markers are related to satellite cell surface, myogenic development and cell proliferation. Statistical analyses were performed using the Rest software.

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5. Conclusão

Em conjunto, esses dados enfatizam a importância da investigação de RGMa no tecido muscular.

No 1º. capítulo:

- A injeção intramuscular de RGMa em camundongos adultos é capaz de induzir hipertrofia muscular, tanto em animais saudáveis como em animais que apresentaram uma lesão muscular prévia.
- Dessa forma, a injeção intramuscular de RGMa parece ser uma boa estratégia para se reverter quadros musculares patológicos como atrofias e distrofias musculares.

No 2º. Capítulo:

- RGMa, uma proteína ancorada à membrana plasmática, está presente no núcleo das células tronco do sistema muscular, ou das células satélites.
- A localização nuclear é provavelmente dependente da via de sinalização BMP.
- RGMa presente no núcleo parece ser capaz de ativar genes relacionados com a proliferação das células satélites.

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7. Anexos

7.1 Atividades de pesquisa

Desde a seleção para entrada no doutorado (agosto/2018), colaborei com a produção de 10 artigos científicos: dois (capítulo 1 e capítulo 2) foram apresentados ao longo dessa tese, dois (1 e 2) estão envolvidos com a linha de pesquisa desenvolvida no laboratório, um (3) está envolvido com o projeto de cultivo de carne *in vitro* que vou desenvolver durante o posdoc, dois (4 e 5) estão envolvidos com outras linhas de pesquisa do nosso laboratório e os outros 3 (6, 7 e 8) são fruto de colaborações com outros grupos pertencentes ao Programa de Pós-graduação em Ciências Morfofuncionais e de Bioengenharia da UFSJ e ao Programa de Pós Graduação em Biologia Celular da UFMG.

1. Manuscrito **publicado**: Aline Goncalves Lio Copola, Iria Gabriela Dias dos Santos, Luiz Lehmann Coutinho, Luiz Eduardo Vieira Del-Bem, Paulo Henrique de Almeida Campos-Junior, Izabela Mamede Costa Andrade da Conceicao**, Julia Meireles Nogueira**. Alinne do Carmo Costa, Gerluza Aparecida Borges Silva and Erika Cristina Jorge. **Transcriptomic characterization of the molecular mechanisms induced by RGMa during skeletal muscle nuclei accretion and hypertrophy.** BMP Genomics, v. 23:188, 2022.

2. Manuscrito **publicado**: Alinne do Carmo Costa, Aline Gonçalves Lio Copola, Clara Carvalho e Souza, **Júlia Meireles Nogueira**, Gerluza Aparecida Borges Silva, Erika Cristina Jorge. **RGMa-Neogenin promotes *in vitro* skeletal muscle cell hyperplasia via the induction of *Myod* expression**. In vitro Cellular & Developmental Biology, v 57: 415-427, 2021.

3. Manuscrito intitulado **Bioactive cellulose acetate nanofiber loaded with annatto support skeletal muscle cell attachment and proliferation** de autoria Ana Elisa Antunes dos Santos, Tiago Cotta, João Paulo Ferreira Santos, Juliana Sofia Fonseca Camargos, Ana Carolina Correia, Erika Gabriele Alves Alcântara, Claudia Fleck, Aline Gonçalves Lio Copola, **Júlia Meireles Nogueira**, Gerluza Aparecida Borges Silva, Luciana Oliveira Andrade, Roberta Ferreira, Erika Jorge foi **submetido** a revista Frontiers in Bioengineering and Biotechnology, section Tissue Engineering and Regenerative Medicine

4. Manuscrito intitulado **Acute exposure to two biocides causes morphological and molecular changes in the gill ciliary epithelium of the invasive golden mussel *Limnoperna fortunei* (Dunker, 1857)** de autoria Amanda Maria Siqueira Moreira, Erico Tadeu Fraga Freitas; Mariana de Paula Reis**; Júlia Meireles Nogueira**; Newton Pimentel de Ulhôa Barbosa; André Luiz Martins Reis; Afonso Pelli; Paulo Ricardo da Silva Camargo; Antonio Valadão Cardoso; Rayan Silva de Paula; Erika Cristina Jorge está pronto para ser **submetido**

5. Manuscrito intitulado **Standardization of loop-mediated isothermal amplification (LAMP) assay for *Limnoperna fortunei* detection** e de autoria de Paula, Rayan; Monte-Neto, Rubens; Wallau, Gabriel; Reis, Mariana; Souza, Clara; **Nogueira, Júlia**; Cardoso, Antonio; Jorge, Erika está pronto para ser **submetido** na revista Biological Invasions

6. Manuscrito intitulado **Drastic loss of antral follicles due to gene expression dysregulation occurs on the first day after subcutaneous ovarian transplantation** e de autoria de Bárbara Rodrigues, Danielle Storino, Ana Paula Madureira, Guilherme Gouveia, Luciola Barcelos, Rayan Silva de Paula, **Julia Nogueira**, Erika Jorge, Paulo Campos Junior foi **aceito** para publicação (RESC-D-22-01030R1) na revista Reproductive Sciences

7. Manuscrito **publicado**: Grazielle A de Sá; Anna Clara P M dos Santos; **Júlia M Nogueira**; Diogo M dos Santos; Flávio A Amaral; Erika C Jorge; Marcelo V Caliari; Celso M Queiroz-Junior, Anderson J Ferreira. **Angiotensin II triggers cartilage and bone lesions in experimental osteoarthritis** Bone, v 145, 2021.

8. Manuscrito **publicado**: Valadão, Priscila Aparecida Costa De Aragão, Bárbara Campos; Andrade, Jéssica Neves; Magalhães-Gomes, Matheus Proença S.; Foureaux, Giselle; Joviano-Santos, Julliane Vasconcelos; Nogueira, José Carlos; Machado, Thatiane Cristina Gonçalves; De Jesus, Itamar Couto Guedes; **Nogueira, Julia Meireles**; De Paula, Rayan Silva; Peixoto, Luisa; Ribeiro, Fabíola Mara; Tapia, Juan Carlos; Jorge, Erika Cristina; Guatimosim, Silvia; Guatimosim, Cristina. **Abnormalities in the Motor Unit of a Fast-Twitch Lower Limb Skeletal Muscle in Huntington's Disease**. ASN Neuro, v. 11, p. 1-20, 2019.

Participação em evento internacional:

- Participação no **EMBO Practical Course on Developmental Biology** da MBL durante 15 dias que aconteceu em Quintay, no Chile em janeiro 2023.
- Participação e apresentação do trabalho intitulado: "**Changes in the Repulsive Guidance Molecule A (RGMa) expression in skeletal muscle cells and muscle stem cells of a Parkinson's Disease murine model**" no Latin American Society for Developmental Biology (LASDB) Meeting na Argentina em 2019.

Participação em evento nacional:

- Participação e apresentação do trabalho intitulado: "**Injeção intramuscular da molécula orientadora por repulsão a (RGMa) induz hipertrofia na musculatura esquelética de camundongos**" no IV Encontro de Morfofisiologia: Inovações tecnológicas e aspectos terapêuticos de 2021.
- Participação e apresentação do trabalho intitulado "**Intramuscular injection of RGMa induces hypertrophy in the skeletal musculature of mice**" no Genética 2021 - Brazilian Congress of Genetics.
- Participação e apresentação do trabalho intutulado: "**Changes in the Repulsive Guidance Molecule A (RGMa) expression in skeletal muscle cells and muscle stem cells of a Parkinson's Disease murine model**" no 65th Congresso Brasileiro de Genética de 2019.
- Participação da **Mostra ICB UFMG** 2019 e jurada do Concurso de Divulgação Científica – modalidade Science Slam.
- Participação e Comissão Organizadora da **Aula Magna da Pós-Graduação do ICB** UFMG 2020 sobre o tema "Mudanças Globais e o Futuro das Florestas Tropicais" ministrada pelo Prof. Jos Barlow (Lancaster, Reino Unido).
- Participação e Comissão Organizadora da **Aula Magna da Pós-Graduação do ICB** UFMG 2021 sobre o tema "Epidemiologia de viroses

emergentes, com ênfase na disseminação do SARS-CoV-2 e suas variantes no mundo" ministrada pelo Prof Túlio de Paiva Nazareth Andrade de Oliveira (UKZN, África do Sul).

- Participação da comissão Organizadora da **Aula Magna da Pós-Graduação do ICB** UFMG 2022 sobre o tema "Parasitos intracelulares, lisossomos e reparo de membranas: uma aventura inesperada em biologia celular" ministrada pela Profa Norma Windsor Andrews.
- Participação e organização do GeneTime Conference 2022, pela PPG Genética – ICB/UFMG Campus Papulha

<u>Participação em projetos paralelos do nosso laboratório:</u>

Resumos apresentados na Semana do Conhecimento, UFMG (15/10/2018 a 19/10/2018):

- Título: "'Análise da viabilidade e adesão de mioblastos imortalizados da linhagem C2C12 cultivados sobre scaffolds de nanofibras de acetato de celulose"
- Título: "Avaliação dos mecanismos moleculares envolvidos na indução da hipertrofia muscular pela molécula orientadora por repulsão RGMa"
- Título: "Avaliação dos efeitos da superexpressão da molécula orientadora por repulsão a1 (RGMa1) em fibroblastos de embriões de galinha *in vitro*"
- Título: "RGMa e RGMb são reprimidos na diferenciação osteogênica terminal"
- Título: **"**RGMa1 induz a expressão de Six1 e MyoD em células de somitos de embriões de galinha"

Resumos apresentados na Semana do Conhecimento, UFMG (2020):

- Título: "RGMa recombinante induz a diferenciação e o crescimento de células musculares *in vitro*."
- Título: "Avaliação da expressão de RGMa nas fibras musculares esqueléticas em um modelo murino de simpatectomia química"

Banca de Trabalho de Conclusão de Curso

- Parecerista na banca de defesa de monografia de conclusão de curso de Ciências Biológicas/UFMG do aluno Ricardo Nodari em 27 de junho de 2019 intitulada “Identificação e caracterização in silico de elementos cis regulatórios do gene de RGMa e sua conservação em vertebrados”.
- Banca avaliadora de defesa de monografia de conclusão de curso de Ciências Biológicas/UFMG da aluna Patrícia Miranda Lima em 08 de julho de 2022 intitulada “A aplicabilidade da Saúde Única para estudar os efeitos dos spillovers de coronavirus de morcegos em humanos e animais e suas repercussões ambientais”.
- Banca avaliadora do trabalho científico intitulado “Radiômica” apresentado em 29 de novembro de 2022 na UMA
- Banca avaliadora do trabalho científico intitulado “Diagnóstico laboratorial da leucemia promielocítica aguda” apresentado em 30 de novembro de 2022 na UNA.
- Banca avaliadora do trabalho científico intitulado “Aspectos clínicos e laboratoriais no diagnóstico diferencial da síndrome de cushing” apresentado em 23 de novembro de 2022 na UNA.
- Banca avaliadora do trabalho científico intitulado “Exames toxicológicos em motoristas profissionais: uma revisão bibliográfica acerca do uso de psicoativos e seus contextos” apresentado em 23 de novembro de 2022 na UNA.
- Banca avaliadora do trabalho científico intitulado “Uso abusivo de opioides: aspectos clínicos e toxicológicos do fentanil¨ apresentado em 23 de novembro de 2022 na UNA.
- Coorientadora do trabalho de conclusão de curso de Ciências Biológicas/UFMG da aluna Jade Carceroni de Sousa Carvalho defendido em 16 de dezembro de 2022 intitulado “Avaliação do padrão de expressão de RGMa nas fibras musculares em um modelo murino de simpatectomia química”.

7.2 Atividades de extensão

- Participei do projeto de extensão “Modelos tridimensionais de Embriologia: Uma produção didática da UFMG como instrumento de transformação e impacto na formação docente e discente por todo país” (SIEX 400522) coordenado pela Profa. Dra. Gerluza Aparecida Borges Silva (PPG-BioCel), cujo objetivo é disponibilizar para a(s) escola(s) participante(s) um método imersivo de ensinar Embriologia atrelada a importantes aspectos da sexualidade pré-adolescente. Este trabalho foi reconhecido e premiado em três eventos distintos:
    - Menção Honrosa, I Jornada Científica de Ensino Extensão, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. 2018.
    - Melhor Trabalho na Categoria Extensão, 4o Encontro de Ciência, Ensino e Cultura do ICB/UFMG, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. 2018.
    - Menção Honrosa de Extensão, XXII Encontro da Extensão/PROEX, Semana do Conhecimento, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. 2019.
- Participação em atividade apresentada ao Programa de Mestrado Profissional em Ensino de Biologia em Rede Nacional (PROFBIO), na disciplina Obrigatória do tema 1, ministrada no dia 29/10/2018. Titulo: “Circuito Dia da Vida: Estratégias didáticas para a conscientização dos riscos da precocidade sexual entre adolescentes de escolas públicas”
- Participei (2017-2018) do Projeto de Extensão “Curious Minds” (SIEX 402913) coordenado pela Profa Dra. Walderez Ornelas Dutra (PPG-BioCel), cujo objetivo foi inserir alunos do ensino médio à vivência laboratorial por meio do desenvolvimento do pensamento científico e de realização de práticas experimentais de forma ativa. Esse projeto resultou em publicação de um artigo científico na Revista MultiAtual (ISSN 2675-4592), em 2020.
- Participei da administração e co-autoria do canal de Divulgação Científica “Trem Bão é Ciência” nas redes sociais Instagram, Facebook e Twitter.

- Participei do II divulgação: Jornada de Divulgação Científica, fui tutora da parte prática do evento e ganhamos o primeiro lugar do hackathon como trabalho intitulado “Circiências” em maio de 2021.
- Participei do II Circuito de Palestras na UNA como palestrante sobre Divulgação Científica em 2021

7.3 Atividades de ensino

- Cursei a disciplina Experiência Didática I e II sob supervisão da Profa Adriana Abalen, onde acompanhei, ministrei aulas teóricas e práticas, além de outras atividades durante o primeiro e o segundo semestre ano de 2019, durante a disciplina “Genética e Nutrição” – BIG 018
- Ministrei o curso à distância “Tópicos Especiais em Biologia Molecular” para a Pós-Graduação em Medicina Veterinária da Universidade de Uberaba – MG, em novembro de 2020.
- Fui beneficiada do programa para o Desenvolvimento do Ensino de Graduação no projeto “Inclusão pedagógica: salas de aula equitativas no ensino superior” Coordenado pela Profa Juliana Estanislau durante 3 semestres, de 10/21 a 02/23 e o trabalho apresentado na Semana do Conhecimento da UFMG de 2022 foi premiado como Relevância Acadêmica
- Durante os dois semestres de 2022 fui Professora Voluntária no Setor de Embriologia do Departamento de Morfologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. Ministrei aulas de Embriologia Aplicada à Medicina Veterinária (30h) para 4 turmas diferentes do curso de Medicina Veterinária.