Transcriptomic Characterisation of the Molecular Mechanisms Induced by RGMa During Skeletal Muscle Hyperplasia and Hypertrophy

Aline Gonçalves Lio Copola
Universidade Federal de Minas Gerais

Íria Gabriela Dias dos Santos
Universidade Federal de Minas Gerais

Luiz Lehmann Coutinho
Universidade de São Paulo

Luiz Eduardo Vieira Del Bem
Universidade Federal de Minas Gerais

Paulo Henrique de Almeida Campos Junior
Federal University of São João del-Rei

Júlia Meireles Nogueira
Universidade Federal de Minas Gerais

Aline do Carmo Costa
Universidade Federal de Minas Gerais

Gerluza Aparecida Borges Silva
Universidade Federal de Minas Gerais

Erika Cristina Jorge (✉ ecjorge@ufmg.br)
Universidade Federal de Minas Gerais

Research Article

Keywords: Axon Guidance, myogenesis, hypertrophy, hyperplasia, skeletal muscle differentiation, transcriptomic analysis

DOI: https://doi.org/10.21203/rs.3.rs-646954/v1

License: ©  This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

Background: The repulsive guidance molecule a (RGMa) is a GPI-anchor axon guidance molecule first found to play important roles during neuronal development. RGMa expression patterns and signalling pathways via Neogenin and/or as BMP coreceptors indicated that this axon guidance molecule could also be working in other processes and diseases, including during myogenesis. Previous works have consistently shown that RGMa is expressed in skeletal muscle cells and that its overexpression induces both nuclei accretion and hypertrophy in muscle cell lineages. However, the cellular components and molecular mechanisms induced by RGMa during the differentiation of skeletal muscle cells are poorly understood. In this work, the global transcription expression profile of RGMa-treated C2C12 myoblasts during the differentiation stage, obtained by RNA-seq, were reported.

Results: RGMa treatment could modulate the expression pattern of 2,195 transcripts in C2C12 skeletal muscle, with 943 upregulated and 1,252 downregulated. Among them, RGMa interfered with the expression of several RNA types, including categories related to the regulation of RNA splicing and degradation. The data also suggested that RGMa hyperplasia effects could be due to their capacity to induce the expression of transcripts related to cell-cell adhesion, while RGMa effects on muscle hypertrophy might be due to (i) the activation of the mTOR-Akt independent axis and (ii) the regulation of the expression of transcripts related to atrophy. Finally, RGMa induced the expression of transcripts that encode skeletal muscle structural proteins and members of the signalling pathways associated with GEF and Rho/Rac, common secondary signals of skeletal muscle hypertrophy, and the canonical pathway of the RGMa/Neogenin signalling.

Conclusions: These results provide comprehensive knowledge of skeletal muscle transcript changes and pathways in response to RGMa.

Background

Repulsive guidance molecule a (RGMa) comprises the first repulsive glycoprotein member identified in the family of repulsive guidance molecules[1]. It was originally identified as a repulsive clue in the orientation of axonal growth in the central and peripheral nervous system and as an important target for neuronal survival [2–5] However, RGMa action domains were found to go beyond the processes related to neurogenesis and could be extended to different processes, including the induction of endochondral ossification during skeletal development [6], the suppression of endothelial tube formation [7], and inflammatory responses [8, 9].

These diverse functions can be performed by RGMa because it can signal through different receptors and work as a modular protein. The RGMa C-terminal domain (C-RGMa) harbours a GPI-anchor and presents affinity to the type I transmembrane neogenin receptor [10, 11], which is known as a guidance receptor for migrating neuronal and mesodermal cells [12–14]. This domain also harbours a von Willibrand type D structural domain, containing a GDPH autocatalytic site [15]. The RGMa N-terminal domain (N-RGMa) harbours a signal peptide, an additional neogenin-binding site, and an RGD motif, that is known to be important in cell-cell adhesion processes mediated by integrins [16]. However, RGMa signalling through integrins has not been reported thus far. Notably, N-RGMa presents high affinity to bone morphogenetic proteins (BMP) ligands, making RGMa (and all the members of this family) a modulator of this important signalling pathway [17–20]. N-RGMa shares the same binding site on the BMP ligand with the ectodomain of the BMP type I receptor A (BMP-R1A), meaning that RGM can induce the BMP canonical signalling pathway via activation of Smad 1/5/8. RGMa could also integrate neogenin and BMP signalling cascades [6, 21–23]. Finally, RGMa was recently found to promote astrogliosis and glial scar formation in a rat model of middle cerebral artery occlusion/reperfusion by forming a complex with ALK5 and Smad2/3, which are the main members of the transforming growth factor β1 (TGFβ1) signalling pathway [24].

In previous works, we found RGMa transcripts in the myogenic and satellite cell precursors in the somites during chicken embryonic development [25] and at the sarcolemma and in the sarcoplasm of adult mice muscle cells [26]. RGMa overexpression in C2C12 cells induced the formation of larger myotubes (hypertrophy) with an increased number of myonuclei
(hyperplasia), while its knockdown resulted in the appearance of smaller cells, with a deficient ability to form multinucleated myotubes [26].

Skeletal muscle cell size is known to be determined by the balance between protein and cellular turnover [27–29]. Because of cellular turnover, the skeletal muscle cell grows by myonuclei accretion (hyperplasia), in a process mediated by cell fusion. The increase of myonuclei into myofibers leads to muscle mass expansion due to the higher rate of transcription given the nuclear turnover [30]. Muscle hyperplasia is important not only during embryonic development but also during muscle regeneration [30–37]. In contrast, because of protein turnover, the skeletal muscle cell grows by upregulating protein synthesis pathways, consequently increasing the level of protein within the muscle tissue [28, 30]. Although hypertrophy and hyperplasia are two distinct processes, they frequently occur together [37], and not all signals involved during the proliferation and differentiation of skeletal musculature are known.

Despite having found that RGMa can induce hypertrophy and hyperplasia of skeletal muscle cells cultivated in vitro, the molecular mechanisms that are induced by this axon guidance molecule in these particular cells have not been investigated thus far. In this work, C2C12 cells were treated with RGMa recombinant protein to investigate the molecular mechanisms that are modulated by this axon guidance molecule during myogenic differentiation. This was the first work to show, through RNA-seq analysis, the transcript targets and molecular profile triggered by RGMa during skeletal muscle differentiation and its possible involvement in multiple functions, including cell fusion and hypertrophy.

**Results**

**Overview of the RNA-seq data and differentially expressed transcripts (DETs)**

The quality of the generated sequence database was first evaluated to verify the internal consistency and reproducibility of the replicate samples, as well as the disparity among them. The Pearson correlation coefficient (PCC) of the normalised read-counts revealed a perfect positive linear correlation between all RGMa-treated samples and an extremely strong correlation among the control ones (Fig. 1A). The analysis also revealed a subtle difference between treated and control samples, as there was a positive linear correlation showing Pearson $r$ coefficients above 0.97 among all correlated samples (Fig. 1A). MA-plot analysis revealed that RGMa treatment modulated gene expression in skeletal muscle cells, with very few of them presenting a drastic effect (Fig. 1B).

The expression of 23,855 transcripts could be detected after normalisation, and 2,195 were found as differentially expressed transcripts (DETs, $p < 0.05$, Fig. 1B, grey dots), with 943 upregulated and 1,252 downregulated by RGMa treatment compared to the control (Fig. 1B, blue dots with $\text{Log2(FC)} > 0$ and $\text{Log2(FC)} < 0$, respectively). Twenty-six DETs were exclusively expressed in RGMa-treated myoblasts, and 79 DETs had their expression drastically altered by the treatment (Supplementary Table 1). The most drastic effects among the DETs were also observed as a heatmap of transcripts with enriched muscle-associated terms (Fig. 1C). The heatmap also allowed the observation that the expression of the majority of the transcripts did not change considerably between the control and treated samples. The 20 most upregulated and 20 most downregulated transcripts by RGMa treatment in C2C12 cells are shown in Table 1.

The most highly upregulated DET induced by RGMa treatment was the *Pou2F1* transcription factor (isoform Pou2F1-208, ENSMUST00000160260.9), also known as Oct-1 (Table 1). Among the other highly expressed genes, RGMa was able to induce the expression of genes related to skeletal muscle structure, including sarcomere and costamere organisation (e.g., *Ank3, Nrap* and *Parva*), vesicle formation and trafficking (e.g., *Myo5a, Iqsec1, Tbc1d25, Acap* and *Rab1a*), and control of the cell cycle (*Mau2* and *Scaper*).

Notably, another isoform of the *Pou2F1* transcription factor (Pou2F1-205, ENSMUST0000011427.9) was found to be one of the most downregulated genes by RGMa treatment (Table 1).
Table 1
The most drastically altered transcripts in rcRGMa-treated C2C12 myoblast, during myogenic differentiation

| Transcripts most downregulated by RGMa | Transcripts most upregulated by RGMa |
|---------------------------------------|--------------------------------------|
| **Ensembl Transcript Access** | **Transcript name** | **log2(FC) < 0** | **Ensembl Transcript Access** | **Transcript name** | **log2(FC) > 0** |
| ENSMUST00000113926.7 | Zfx-203 | -12,09700663 | ENSMUST00000160260.8 | Pou2f1-208 | 11,86541962 |
| ENSMUST00000187142.1 | Zfp469-202 | -11,6517089 | ENSMUST00000075836.1 | Dock7-202 | 11,30650103 |
| ENSMUST00000111427.8 | Pou2f1-205 | -11,58532487 | ENSMUST00000182593.7 | Prrc2c-209 | 11,18773896 |
| ENSMUST00000113870.2 | Tsc1-204 | -11,08073619 | ENSMUST00000182155.7 | Myo5a-214 | 9,84258635 |
| ENSMUST00000169353.2 | Kifc3-202 | -10,96464702 | ENSMUST00000040711.1 | Nrap-201 | 10,01424135 |
| ENSMUST00000177916.7 | Zfp131-201 | -10,66135536 | ENSMUST00000106643.7 | Parva-203 | 9,856496026 |
| ENSMUST00000134230.7 | Hnmph1-211 | -10,61576893 | ENSMUST00000155282.8 |  |
| ENSMUST00000107857.10 | Ap2a1-202 | -10,25950022 | ENSMUST00000097864.8 | Pum1-203 | 9,697390548 |
| ENSMUST00000194801.5 | Rbm5-224 | -10,1739189 | ENSMUST00000212451.1 | Mau2-206 | 9,650005064 |
| ENSMUST00000132947.1 | Pds5b-204 | -9,920365984 | ENSMUST00000217647.1 | Scaper-205 | 9,63167893 |
| ENSMUST00000154403.7 | Polg-214 | -9,869641974 | ENSMUST00000212100.1 | Igsec1-210 | 9,270515836 |
| ENSMUST00000170647.1 | Tnpo3-209 | -9,791146902 | ENSMUST00000039892.8 |  |
| ENSMUST00000231973.1 | D16Ertd472e-205 | -9,770163529 | ENSMUST00000183148.7 | Ank3-239 | 9,209545896 |
| ENSMUST00000095037.1 | Whrm-204 | -9,756960752 | ENSMUST00000163483.1 | Rab1a-206 | 9,145085159 |
| ENSMUST00000208730.1 | Picalm-212 | -9,589780073 | ENSMUST00000230614.1 | Acp2-203 | 9,047789318 |
| ENSMUST00000066986.12 | Zfp142-202 | -9,460284761 | ENSMUST00000171937.1 | Arhgap35-202 | 6,619772687 |
| ENSMUST00000222395.1 | Atg2b-205 | -9,411773318 | ENSMUST00000205765.1 | Crebbp-205 | 6,563438613 |
| ENSMUST00000150905.1 | Htra1-204 | -9,36305965 | ENSMUST00000224799.1 | Spire1-207 | 5,741134073 |
| ENSMUST00000092614.8 | Pcgf1-201 | -9,362877176 | ENSMUST00000098816.9 | Slc7a2-202 | 4,478565603 |
| ENSMUST00000216284.1 | Cep164-207 | -4,150389783 | ENSMUST00000194877.5 | Ints7-206 | 4,424334603 |

**Note.** The twenty most highly downregulated (Log2(Fold Change) < 0) and twenty most highly upregulated (Log2(Fold Change) > 0) Differentially Expression Transcripts (DET - with a false discovery rate (FDR) < 0.05) modulated in C2C12 cells treated with RGMa during differentiation. Access number and transcript name identified in the Ensembl database; log2(FD) < 0 corresponds to the fold change of the downregulation and log2(FD) > 0, of the upregulation of each transcript after RGMa treatment.
Among the others, the downregulation of genes from the same categories included those associated with regulators of muscle mass and structure, such as \textit{Tsc1} and \textit{Tnpo3}, with the formation of clathrin-coated vesicles (\textit{Kifc3}, \textit{Ap2a1}, and \textit{Picalm}), and with cell cycle progression (\textit{PCGF1}) (Table 1).

**RNA categories among DET**

Given the reliability of the transcriptome data, we next classified all 2,195 DETs by RNA biotypes to determine which were the main RNA categories influenced by RGMa treatment. Among the 1,252 DETs that were found to be downregulated, 917 (73.2\%) were protein coding, 246 (19.6\%) were processed transcripts, 67 (5.35\%) were NMDs, and 22 (1.75\%) were pseudogenes (Fig. 2). Among the 943 upregulated DETs, 786 (83.3\%) were protein coding, 115 (12.2\%) were processed transcripts, 36 (3.8\%) were NMDs, 5 (0.5\%) were pseudogenes, and 1 (0.1\%) was a TEC (Fig. 2). Overall, this data revealed that most of the RNA biotypes that were modulated by RGMa treatment were ORF-containing RNAs, while the remaining were composed of RNAs mainly associated with the regulation of gene expression, including the NMD category, which was composed of transcripts containing a premature stop codon, and processed transcripts, a category composed of IncRNA, ncRNA, antisense, and intron-retained RNAs.

**GO pathway enrichment analysis of the non-coding RNA found as DETs**

The non-coding RNA found as DETs that were upregulated by RGMa treatment were mostly involved with the ‘regulation of RNA splicing,’ ‘stress fibre,’ ‘myoblast fusion,’ and ‘integrin binding’ (Fig. 3A), while ‘regulation of RNA transport’ and ‘peptide biosynthetic process’ were enriched among the downregulated non-coding DETs (Fig. 3B).

**GO pathway enrichment analysis of the protein coding RNA found as DETs**

DETs were characterised based on the Gene Ontology (GO) terms to identify the pathways that were enriched among the up- and downregulated transcripts. The enriched GO terms for the protein coding upregulated DETs were mostly related to the following biological processes: ‘morphogenesis,’ ‘metabolism,’ and ‘developmental regulation of muscle cell’ (Fig. 4A). Related to the cellular components, RGMa treatment could induce the upregulation of transcripts associated with ‘cytoskeleton,’ ‘cell projection,’ ‘endomembrane system,’ ‘adherens junction,’ ‘nucleus,’ and ‘nucleoplasm’ (Fig. 4B); and related to molecular function, transcripts were grouped as ‘nucleic acid binding,’ ‘transcription factor binding,’ and ‘regulation of GTPase’ and ‘Ras GTPase activity’ (Fig. 4C).

A different pattern was found with the classification of the protein coding downregulated DETs; terms related to ‘metabolism’ and ‘tissue survival’ were the most downregulated after RGMa treatment. ‘Purine nucleoside triphosphate metabolic process,’ ‘peptide biosynthetic process,’ ‘translation,’ and ‘positive regulation of apoptotic signalling pathway’ were the most enriched terms of biological processes (Fig. 5A). Cellular components were mostly associated with ‘mitochondria protein complex,’ ‘actin cytoskeleton,’ ‘cytosolic ribosome,’ ‘proteasome complex,’ and ‘vesicle coat’ (Fig. 5B), while those found to be associated with molecular function were grouped in ‘activity of nucleoside-triphosphatase,’ ‘ATPase,’ and ‘positive regulation of catalysis’ categories (Fig. 5C).

**Enriched muscle-associated terms**

In general, upregulated DETs from all RNA categories were associated with the following enriched terms: ‘muscle development,’ ‘muscle differentiation,’ ‘muscle morphology,’ ‘muscle structure,’ ‘muscle adaptation,’ ‘muscle migration,’ ‘proliferation,’ ‘growth,’ and ‘hypertrophy’ (Fig. 6A). Downregulated DETs were enriched with the terms ‘tropomyosin and troponin binding,’ ‘myofibril,’ ‘sarcomere and contractile fibre,’ ‘muscle proliferation,’ ‘development,’ and ‘migration,’ as well as ‘differentiation of striated, cardiac, and smooth muscleature’ (Fig. 6B).

**RNA-seq validation**

We chose 12 DET isoforms to validate our RNA-seq data and analysis by qPCR. From the upregulated DETs, \textit{Arhgap-35}, \textit{Hipk2}, \textit{Kifc3}, \textit{Mef2d}, \textit{mTOR}, \textit{Myh9}, \textit{Myo5a}, \textit{Nfat5}, \textit{Parva}, and \textit{Pou2f1-208} were selected. Among the downregulated DETs, \textit{Cep} and
Pcgf were chosen. The qPCR results showed a total concordance with the RNA-seq analysis (Fig. 7).

**Discussion**

Although originally identified as a guidance clue for axonal growth, RGMa has been identified as playing roles in a number of different biological processes, including during myogenesis. RGMa transcripts could be found in chicken somites at the origin site of the muscle and satellite cell precursors [25]. In adult muscle, RGMa was found regions of the sarcolema and sarcoplasm, with an expression pattern similar to sarcomeric proteins [26]. Initial functional studies revealed that RGMa can induce myonuclear accretion and hypertrophy of myotubes, suggesting that this axon guidance molecule might be involved with the mechanisms that modulate skeletal muscle cell size [26]. However, the molecular mechanisms induced by RGMa during these important muscle phenotypes have not been clarified thus far. RGMa exerts its canonical effects through the type-I transmembrane neogenin receptor [7, 8, 10, 38], but it can also work as a bone morphogenetic protein (BMP) co-receptor, as it shares the same binding site in BMP-R1A with BMP ligands [16]. Notably, both signalling pathways seem to be active in skeletal muscle cells, inducing similar phenotypes in controlling the cell size, but these effects were never investigated in the context of having RGMa as a possible ligand. Using RGMa recombinant proteins in C2C12 cells, we could not clearly elucidate if RGMa effects were induced via neogenin and/or BMP signalling pathways, possibly because these receptors do not have RGMa as an exclusive ligand [39]. For this reason, in this work, the transcriptome of C2C12 cells was sequenced after being treated with RGMa recombinant protein during the late differentiation stage to detect the transcripts that had their expression modulated by this axon guidance molecule during the induction of hypertrophy and hyperplasia of skeletal muscle cells.

A database composed of 23,856 transcripts expressed during C2C12 differentiation was generated. Sequenced biological triplicates from treated and control groups were found to be homogeneous, conferring internal consistency and reproducibility of replicate samples. Three biological replicates are considered a sufficient for a reliable quantitative inferential analysis [40]. From these expressed transcripts, 2,195 were modulated by RGMa treatment, with 943 upregulated and 1,252 downregulated. From this database, it was noted that RGMa was able to modulate the expression of five RNA biotypes. The most frequent RNA biotype modulated by RGMa was ‘protein coding,’ which included ORF containing transcripts. However, a significant portion of DETs were included in categories involved with the regulation of gene expression, including in the ‘nonsense mediated decay’ (composed of transcripts with a premature stop codon) and ‘processed transcript’ (composed of ‘retained intron RNA’, ‘antisense’ and ‘ncRNA’) biotypes. According to Wong et al. (2013), a number of transcripts must be destroyed to permit developmental transitions during differentiation [41]. Therefore, this data suggests that RGMa treatment induced the regulation of the genes that were being expressed during the differentiation stages using these particular molecular mechanisms, allowing the adaptation of these cells to reach terminal differentiation.

Additionally, our analysis also revealed that RGMa could differentially induce the expression of alternatively spliced transcripts. Pou2F1 (Pou Class 2 Homeobox 1, also known as Oct-1) isoforms were found to be the most upregulated DETs (Pou2f1-208, ENSMUST00000160260.9), as well as the most downregulated DETs (Pou2f1-205, ENSMUST00000111427.9) by RGMa treatment in skeletal muscle cells. Pou2F1 is a member of the Pou transcription factor family and is associated with a plethora of processes, including the activation of some snRNA, histone H2B, immunoglobulins, and other housekeeping genes [42], the regulation of the circadian clock [43], and glycolytic metabolism [44]. In skeletal muscle cells, this transcription factor was associated with the activation of pro-inflammatory immune response in patients with myalgia [45] and with MyHC IIB expression, when associated with MEF2 and the serum response factor (SRF) [46–48]. Pou2F1 was also identified on a slow skeletal muscle troponin I promoter in Gaoyou duck skeletal muscle [49]. In addition to Pou2F1, multiple isoforms for myoferlin (Myof), myosin heavy chain 10 (Myh10), myosin IXB (Myo9b), titin (Ttn), tensin 2 (Tns2), supervillain (Svil), and chromodomain helicase DNA binding protein 2 (Chd2) were also found as modulated by the RGMa treatment in C2C12 cells; these genes are of wide importance for development, differentiation, and maintenance of skeletal muscle cells.

**RGMa treatment modulated the expression of muscle hypertrophic markers**
The protein coding DETs were analysed to determine how RGMa induces hypertrophic and hyperplasic effects on skeletal muscle cells.

Our transcriptome database showed that RGMa induced the expression of the mammalian target of rapamycin (mTOR) transcript, which is a common factor from different pathways that culminate with skeletal muscle hypertrophy [28, 50–54]. RGMa could specifically induce mTOR transcript isoform 202 (ENSMUST00000103221.10), suggesting a new mechanism for this isoform in these cells. The effect of RGMa on mTOR expression was also confirmed by qPCR. mTOR exerts its effects as part of two complexes, termed mTORC1 and mTORC2. Increased mTORC1 activity can positively regulate muscle protein synthesis via S6K1 and also inhibit its negative regulation when working via 4EBP1 [28, 55]. TSC1, in a complex with TSC2, is responsible for the negative regulation of mTORC1 signalling, inhibiting the nutrient-mediated or growth factor-stimulated phosphorylation of S6K1 and 4EBP1 [54]. Furthermore, TSC1-204 (ENSMUST00000113870.3) was also highly downregulated by RGMa treatment in skeletal muscle cells. The inhibition of TSC1/2 protein synthesis resulted in rapid activation of mTORC1 signalling independent of Akt [54, 56]. The hypertrophic effects observed by RGMa treatment could then be a result of the inhibition of the TSC1 transcript and of the induction of mTOR expression, which are both crucial for muscle growth.

Additionally, although the TSC1/2 complex is not physically associated with mTORC1, it is required for mTORC2 activation and consequently, for Akt phosphorylation, in a manner that is independent of its GTPase-activating protein activity toward Rheb [57]. Thus, the inhibition of TSC1 by RGMa suggests that RGMa simultaneously works to prevent mTORC2 activation. The fact that TSC1 inhibition contributes to mTORC1 activation independently of Akt, as well as to mTORC2 inhibition, resulting in the loss of Akt stimulation [56], might explain why Akt was not induced by RGMa in skeletal muscle cells. Our results suggest that mTOR upregulation in response to RGMa is independent of Akt phosphorylation.

Other factors associated with the mTORC pathway were also dysregulated by the RGMa treatment and could contribute to including this axon guidance molecule in an alternative muscle hypertrophic pathway. For example, RGMa could induce the expression of the phospholipase D1 (Pld1-202, ENSMUST00000120834.8) transcript, which was found to be an activator of mTORC1 [51, 58].

RGMa could also induce the upregulation of members of the Myocyte Enhancer Factor 2 (Mef2) family, specifically Mef2a-204 (ENSMUSG000000030557.17) and Mef2d-204 (ENSMUSG0000001419.17) isoforms. Mef2 transcription factors activate many muscle-specific growth factor-induced genes and regulate muscle cell differentiation and muscle embryonic development [59–61]. Mef2 can also act as a nodal point for remodelling programs in metabolic gene expression, fibre-type switching, and skeletal muscle regeneration [59, 60, 62]. Mef2a upregulation can also contribute to terminal differentiation and myoblast fusion, which is also consistent with the present GO term analysis and with the RGMa muscle phenotype [26, 39]. Mef2a, Mef2c, and Mef2d deleted in combination in satellite cells abolished skeletal muscle regeneration after cardiotoxin injury [60].

Our RNA-seq database suggested other hypertrophic mechanisms that could be regulated by RGMa treatment, including the upregulation of Sirtuin 1 (Sirt1), which is known to regulate protein degradation via FoxO inhibition [63]; the upregulation of Nos1, which interacts with Sirt1 [64]; or the downregulation of genes that promote muscle protein degradation, such as the activating transcription factor 4 (Atf4) [65, 66].

RGMa treatment also modulated the expression of genes associated with cell fusion.

We have also searched for genes associated with hyperplasia or myonuclear accretion that were modulated by RGMa treatment in C2C12 cells. Among these, cadherin2 (Cdh2, ENSMUST00000025166.13), integrin alpha-V (Itgav, ENSMUST00000141725.2), neural cell adhesion molecule (NCAM, ENSMUST000001668.11), calcium voltage-gated channel subunit alpha1S (Cacna1s, ENSMUST0000012068.9), actinin alpha 1 (Actn1, ENSMUST00000167327.1), disabled homolog 2 (Dab2, ENSMUST00000080880.11), myoferlin (Myo10, ENSMUST0000014175.15, ENSMUST00000224518.1), the myosins Myo5a (ENSMUST00000155282.8, ENSMUST00000123128.7), Myo10 (ENSMUST0000022882.11, ENSMUST0000110457.7, ENSMUST0000125667.2), Myh9 (ENSMUST0000016771.12), Myh10 (ENSMUST00000102611.9) phosphatase, and actin regulator 4 (Phactr4, ENSMUST0000136711.7) were upregulated by RGMa treatment.
**Myof**, for example, is a member of the Ferlin protein family, highly expressed in myoblasts during the pre-fusion phase of differentiation and in myofibers, especially during regeneration after injury [67–69]. It is associated with fusion events and intracellular trafficking in muscle, including myoblast fusion, vesicle traffic, membrane repair, and endocytic recycling [70].

**Myh9** and **Myh10** are equally fundamental for the positive regulation of cell-cell adhesion and myoblast fusion [71]. **Myh9** is known as non-muscle myosin heavy chain IIa (NMMHC-IIa), while **Myh10** is the non-muscle myosin heavy chain IIb [72]. These myosins are expressed in most cell types, working as motor proteins in a variety of processes requiring contractile force, such as cytokinesis, cell migration, polarisation and adhesion, maintenance of cell shape, and signal transduction [73–75]. In skeletal muscle cells, non-muscle myosins drive myoblasts to align and fuse to form multinucleated myotubes [71, 76]. The knockdown of these myosins inhibit the change of the myoblast shape, interfering with cell-cell adhesion and fusion [71].

**Dab2** plays an important role as a modulator of cell-cell interactions, as it is a clathrin adaptor and can mediate integrin signalling [77]. In the musculature, **Dab2** was detected during early myogenic differentiation [78, 79]. Shang et al. (2020) showed that **Dab2** expression is upregulated in C2C12 myoblast during the differentiation in myotubes, and its knockdown resulted in reduced myoblast fusion and fewer myotubes. Besides, **Dab2** overexpression could enhance the myotube formation and also restore the myotube differentiation capacity of its knockdown [80].

The **calcium voltage-gated channel subunit alpha1 S** (Cacna1s-202, ENSMUST00000112068.10) encodes one of the five subunits of the L-type voltage-dependent calcium channel in skeletal muscle cells. In the musculature, calcium is generally related to muscle contraction and muscle relaxation [81–83]. However, the regulation of calcium influx into muscle cells plays a critical role in muscle differentiation [83, 84]. Intracellular calcium is able to regulate transcription factors necessary for myotube fusion [84, 85], while its reduction inhibits myoblast differentiation [86]. The upregulation of **Cacna1s** in response to RGMa treatment suggests an association with the regulation of intracellular calcium, which is important for the myoblast fusion process and myotube contraction.

**Conclusion**

The current work allowed us to unravel some molecular mechanisms that were altered in skeletal muscle cells after treatment with RGMa, especially those associated with muscle hyperplasia and hypertrophy. Our analysis suggested that RGMa induced cell hypertrophy via (i) upregulation of hypertrophic markers, (ii) downregulation of inhibitors of hypertrophic pathways, (iii) downregulation of transcripts related to the positive regulation of muscle atrophy, and (iv) upregulation of transcripts that negatively regulate atrophy. At the same time, transcripts associated with known hyperplasic pathways were also found to be modulated by RGMa, mainly those related to cell-cell adhesion pathways.

Our results provide comprehensive knowledge of skeletal muscle transcriptional changes and pathways in response to RGMa treatment.

**Material And Methods**

**Cell culture and differentiation**

The lineage of immortalised mouse myoblasts C2C12 (ATCC® CRL1772™) was cultured at 37°C and 5% CO₂ in growth medium (GM), composed of DMEM (Dulbecco's Modified Eagle's Medium) with high glucose and L-glutamine (Gibco), supplemented with 10% foetal bovine serum (FBS, Gibco) and 1% penicillin, streptomycin, and amphotericin B solution (Gibco). Myogenic differentiation was induced in differentiation medium (DM), composed of DMEM, supplemented with 2% horse serum (Gibco) and 1% penicillin, streptomycin, and amphotericin B. For growth or differentiation conditions, the medium was replaced every 2 days.

**RGMa recombinant protein treatment**
C2C12 cells were seeded at 2×10^4 cells per well in 24-well plates and cultivated in GM at 37°C and 5% CO₂. After reaching 90–100% confluency, cells were induced to differentiate in DM for 72 h. DM was then replaced with fasting medium (FM), composed of DMEM supplemented with 0.2% FBS, and cells were incubated at the same conditions for 3 h. Subsequently, C2C12 were treated with 50 ng/ml mouse RGMa recombinant protein (R&D Systems) in FM and incubated for an additional 48 h, as previously described [39]. The recombinant protein was omitted in the control samples.

**Total RNA isolation and cDNA synthesis**

Cells were harvested in TriReagent (Sigma Aldrich) as pools of three wells in triplicate. Total RNA isolation was performed according to the manufacturer's instructions. Sample integrity, purity, and concentration were evaluated by electrophoresis in 1% agarose gel and in NanoDrop® ND-1000 UV/Vis Spectrophotometer, respectively.

The quality of the total RNA was also evaluated in a Bioanalyzer (Agilent) before being submitted to sequencing. Values for RNA integrity number (RIN) ranging from 8 to 10 were considered suitable for RNA-seq.

**RNA-seq library preparation and next-generation sequencing (NGS)**

For cDNA library construction, 2 µg of total RNA were treated with 1U of DNaseI amplification grade (Invitrogen) and purified according to the Illumina protocol (http://grcf.jhmi.edu/hts/protocols/mRNA-Seq_SamplePrep_1004898_D.pdf), using magnetic microspheres for messenger RNA separation. The purified mRNA was fragmented in Illumina buffer. Superscript III (Invitrogen) and oligo(dT) were used for reverse transcription of the first cDNA strand. The second strand was synthesised using the enzymes RNase H and DNA Polymerase I (Illumina). Molecule ends were treated with T4 DNA Polymerase and Klenow DNA Polymerase (Illumina), making them blunt. The 3’ end of the synthesised cDNA was phosphorylated with T4 PNK (Illumina) and adenylated with Klenow exo (Illumina). Adaptors were bound to cDNA ends, and the samples were purified and selected by size of 200 bp ± 25 bp after fractioning in agarose gel electrophoresis (QIAquick Gel Extraction Kit, QIAGEN). Purified cDNA was quantified by RT-qPCR using adaptor-specific oligonucleotides (Illumina).

Sequencing was performed using Illumina HiSeq 2500, according to the manufacturer's recommendations, and using the paired-end reads protocol. Each sample was sequenced until it reached around 34 million reads/library.

**Mapping RNA-seq data**

Transcript quantification analysis was performed based on Salmon (version 0.13.1), an open-source and freely-licensed software (available at https://github.com/COMBINE-lab/Salmon [87]). Raw reads were used as an input to quantify transcripts in mapping-based mode. The current version of the mouse transcriptome (available at https://www.gencodegenes.org/mouse/release_M20.html) was used as a reference.

**Statistical RNA-seq**

Statistical analysis was performed using the DESeq2 package of R Bioconductor [88]. An adjusted p-value with a false discovery rate (FDR) correction (Benjamini and Hochberg, 1995) of 5% was calculated and used to control false-positive significance in transcript expression variation. Log2(fold change) > 0 and log2(fold change) < 0 were selected as the threshold to show an increase or decrease in transcript expression of treated groups relative to the control group.

**Transcript expression pattern and RNA-seq quality analysis**

Transcript-specific normalisation was performed to remove disparities in the base means correlations and to eliminate the noise of transcripts with low expression.

Normalised transcripts were plotted in MA form using the DESeq2 package to generate a scatter plot of log2 fold changes < 0 and > 0 versus the mean of normalised counts of transcripts, considering DE those with FDR < 0.05. The correlation of each sample and the clustering of the treated and control groups was performed by calculating the PCC of normalised read-counts.

**Functional RNA-seq analysis**
The GO enrichment analysis of DETs was performed using the plugin ClueGO v.2.5.4 [89] for Cytoscape v3.7.1 [90]. The node colours represent the functional groups, and the node size represents the term enrichment significance. Only the most significant term in the group was labelled. The edges connecting the nodes were based on the Kappa statistic (Kappa Score Threshold of 0.4). The right-sided hypergeometric test was used to identify overrepresented GO terms, and the Benjamini-Hochberg method was used for the correction of the p-values (p < 0.001). The functionally grouped network of enriched categories for DETs was annotated for GO terms (cellular component, biological process, molecular function, and immune system process) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) terms. The Ensembl Transcript ID of the DETs was used as input for ClueGO analysis.

The heatmap graph was obtained using the D3Heatmap package (https://www.rdocumentation.org/packages/d3heatmap/versions/0.6.1.2), using ID ensemble transcripts as an input (of clue go output for muscle associated terms) and the correlated base mean expression.

**Primer design and qPCR**

qPCR was performed for twelve up-(Arhgap-35, Hipk2, Kifc3, Mef2d, mTOR, Myh9, Myo5a, Nfat5, Parva, Pou2f1-208) and downregulated (Cep and Pcgf) DETs that were selected due to their importance for muscle phenotypes, as well as by their relevance between the more enriched terms.

Before primer design, the multiline interface (http://multalin.toulouse.inra.fr/multalin/) was used for the alignment of different isoforms of the same gene. The non-consensus sequences were selected to obtain an amplicon of up to 250 bp. Primer 3.0 software was used for primer design. Manual primers were designed for small specific strings.

qPCR was performed in the Rotor-Gene RT-qPCR system (Qiagen), using the iTaq Universal Sybr Green Supermix (Bio Rad) and 0.4–0.8 µM of each primer for a final volume of 10 µl. GAPDH was used as a housekeeping gene. The analysis of differential gene expression was performed using REST 2009 (Relative Expression Software Tool, V.2.0.13) software via randomisation tests (Pair Wise Fixed Reallocation Randomisation Test) [91] with 95% significance.

**Abbreviations**

4EBP1: Eukaryotic Translation Initiation Factor 4E Binding Protein 1;

Acap: ArfGAP With Coiled-Coil, Ankyrin Repeat And PH Domains 1

Actn1: Actinin alpha 1;

Akt: Serine/Threonine Kinase 1;

ALK5: Activin A Receptor Type II-Like Protein Kinase Of 53kD, also Known as Transforming Growth Factor Beta Receptor 1;

Ank3: Ankyrin 3;

Ap2a1: Adaptor Related Protein Complex 2 Subunit Alpha 1;

Arhgap-35: Rho GTPase Activating Protein 35;

Atf4: Activating transcription factor 4;

BMP: Bone Morphogenetic protein;

BMP-R1A: BMP type I receptor A;

C2C12: Immortalized Mouse Myoblast cell line;

Cacna1s: Calcium voltage-gated channel subunit alpha1S;
Cep164: Centrosomal Protein 164;
Cdh2: cadherin2;
cDNA: complementary DNA;
Chd2: Chromodomain helicase DNA binding protein 2;
C-RGMa: RGMa C-terminal domain;
Dab2: Disabled homolog 2;
DE: Differentially expressed;
DET: Differentially expressed transcripts;
DM: Differentiation medium;
DMEM: Dulbecco's Modified Eagle's Medium;
FBS: Fetal bovine serum;
FDR: False Discovery Rate;
FM: Fasting medium;
FoxO: Forkhead Box Protein O;
GDPH: Glyceraldehyde 3-phosphate dehydrogenase;
GM: Growth medium;
GO: Gene Ontology;
GPI-anchor: Glycosylphosphatidylinositol-anchor;
GTP: Nucleotide guanosine triphosphate;
Hipk2: Homeodomain Interacting Protein Kinase 2;
Iqsec1: IQ Motif And Sec7 Domain ArfGEF 1;
Itgav: Integrin alpha-V;
KEGG: Kyoto Encyclopedia of Genes and Genomes;
Kifc3: Kinesin Family Member C3;
IncRNA: Long non coding RNA;
Mau2: MAU2 Sister Chromatid Cohesion Factor;
Mef2: Myocyte Enhancer Factor 2;
mRNA: Messenger RNA;
mTOR: Mechanistic Target Of Rapamycin Kinase;
mTORC1/2: mechanistic target of rapamycin complex 1/2;
Myh9: Myosin heavy chain 9;
Myh10: Myosin heavy chain 10;
Myof: Myoferlin;
Myhc IIB: Myosin heavy chain IIB;
Myo5a: Myosin VA;
NCAM: Neural cell adhesion molecule;
NCBI: National Center for Biotechnology Information;
ncRNA: Non coding RNA;
NGS: Next Generation Sequencing;
NMD: Nonsense Mediated Decay;
Nos1: Nitric Oxide Synthase 1;
Nrap: Nebulin Related Anchoring Protein;
N-RGMa: RGMa N-terminal domain;
Oct-1: Octamer-Binding Transcription Factor 1;
Oligo(dT): Oligonucleotide (deoxythymine);
ORF: Open reading frame;
Parva: Parvin Alpha;
PCC: Pearson correlation coefficient;
Pcgf1: Polycomb Group Ring Finger 1;
Phactr4: phosphatase and actin regulator 4;
Picalm: Phosphatidylinositol Binding Clathrin Assembly Protein;
Pld1: Phospholipase D1;
Pou2F1: Octamer-Binding Transcription Factor-1, also known as Oct1;
Rab1a: RAB1A, Member RAS Oncogene Family;
Rac: Family Small GTPase 1;
RGD: Tripeptide Arg-Gly-Asp;
RGMa: Repulsive Guidance Molecule a;
Rho: Rhodopsin;
RIN: RNA integrity number;

RNA: Ribonucleic acid;

RNA-seq: RNA sequencing;

RT-qPCR: Quantitative reverse transcription polymerase chain reaction;

S6k1: Ribosomal Protein S6 Kinase B1;

Scaper: S-Phase Cyclin A Associated Protein In The ER, also known as Zinc Finger Protein 291;

Sirt1: Sirtnin;

Smad2/3: Mothers Against Decapentaplegic Homolog 2/3;

snRNA: Small nuclear RNA;

SRF: Serum response factor;

Svil: Supervillain;

T4 PNK: T4 Polynucleotide Kinase;

Tns2: Tensin2;

Ttn: Titin;

Tbc1d25: TBC1 Domain Family Member 25;

TEC: To be Experimentally Confirmed;

TGF β1: Transforming Growth Factor β1;

Tnpo3: Transportin 3;

Tsc1: Tuberous Sclerosis 1 Protein;

Tsc2: Tuberin;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw transcriptome sequencing data (RNA-seq) from the biological replicates of C2C12 myoblast treated with RGMa are available under the NCBI-BioProject submission code PRJNA730936. The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing Interests
The authors declare that they have no competing interests.

**Funding**

This research was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). AGLC, ACC and JMN were financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Finance Code 001. ECJ and LLC received a scholarship from CNPq.

**Author’s Contributions**

For this research, AGLC and ECJ designed the study. AGLC, JMN, and ACC performed the in vitro and qPCR experiments. LLC generated the RNA-seq data, and LEDB and PHAC performed the bioinformatic analysis. AGLC and IGDS extracted the biological information of the expression data. AGLC and ECJ wrote the manuscript. ECJ supervised and administrated the study. GABS provided part of the funding acquisition and supervised experimental work. All authors have read and agreed to the published version of the manuscript.

**Acknowledgements**

We wish to thank Professor Antonio Figueira from Centro de Energia Nuclear na Agricultura (CENA, Universidade de São Paulo, Piracicaba, Brazil) for supporting our laboratory.

**References**

1. Monnier PP, Sierra A, Macchi P, Deitinghoff L, Andersen JS, Mann M, Flad M, Hornberger MR, Stahl B, Bonhoeffer F et al. *RGM is a repulsive guidance molecule for retinal axons*. Nature 2002, **419**(6905):392-395.
2. Stahl B, Müller B, von Boxberg Y, Cox EC, Bonhoeffer F: *Biochemical characterization of a putative axonal guidance molecule of the chick visual system*. Neuron 1990, **5**(5):735-743.
3. Müller B, Jay D, Bonhoeffer F: *Chromophore-assisted laser inactivation of a repulsive axonal guidance molecule*. Current Biology 1996, **6**(11):1497-1502.
4. Monnier PP, Sierra A, Macchi P, Deitinghoff L, Andersen JS, Mann M, Flad M, Hornberger MR, Stahl B, Bonhoeffer F: *RGM is a repulsive guidance molecule for retinal axons*. Nature 2002, **419**(6905):392.
5. Siebold C, Yamashita T, Monnier PP, Mueller BK, Pasterkamp RJ: *RGMs: structural insights, molecular regulation, and downstream signaling*. Trends in cell biology 2017, **27**(5):365-378.
6. Zhou Z, Xie J, Lee D, Liu Y, Jung J, Zhou L, Xiong S, Mei L, Xiong WC: *Neogenin regulation of BMP-induced canonical Smad signaling and endochondral bone formation*. Dev Cell 2010, **19**(1):90-102.
7. Harada K, Fujita Y, Yamashita T: *Repulsive guidance molecule A suppresses angiogenesis*. Biochem Biophys Res Commun 2016, **469**(4):993-999.
8. Fujita Y, Yamashita T: *The roles of RGMA-neogenin signaling in inflammation and angiogenesis*. Inflamm Regen 2017, **37**:6.
9. Nohra R, Beyeen AD, Guo JP, Khademi M, Sundqvist E, Hedreul MT, Sellebjerg F, Smestad C, Oturai AB, Harbo HF et al: *RGMA and IL21R show association with experimental inflammation and multiple sclerosis*. Genes Immun 2010, **11**(4):279-293.
10. Rajagopalan S, Deitinghoff L, Davis D, Conrad S, Skutella T, Chedotal A, Mueller BK, Strittmatter SM: *Neogenin mediates the action of repulsive guidance molecule*. Nat Cell Biol 2004, **6**(8):756-762.
11. Itokazu T, Fujita Y, Takahashi R, Yamashita T: *Identification of the neogenin-binding site on the repulsive guidance molecule A*. PLoS One 2012, **7**(3):e32791.
12. Fitzgerald DP, Seaman C, Cooper HM: *Localization of Neogenin protein during morphogenesis in the mouse embryo*. Dev Dyn 2006, **235**(6):1720-1725.
13. Fitzgerald DP, Cole SJ, Hammond A, Seaman C, Cooper HM: Characterization of neogenin-expressing neural progenitor populations and migrating neuroblasts in the embryonic mouse forebrain. *Neuroscience* 2006, 142(3):703-716.

14. Cole SJ, Bradford D, Cooper HM: Neogenin: A multi-functional receptor regulating diverse developmental processes. *Int J Biochem Cell Biol* 2007, 39(9):1569-1575.

15. Bell CH, Healey E, van Erp S, Bishop B, Tang C, Gilbert RJ, Aricescu AR, Pasterkamp RJ, Siebold C: Structure of the repulsive guidance molecule (RGM)-neogenin signaling hub. *Science* 2013, 341(6141):77-80.

16. Healey EG, Bishop B, Elegeheert J, Bell CH, Padilla-Parras S, Siebold C: Repulsive guidance molecule is a structural bridge between neogenin and bone morphogenetic protein. *Nat Struct Mol Biol* 2015, 22(6):458-465.

17. Babitt JL, Zhang Y, Samad TA, Xia Y, Tang J, Campagna JA, Schneyer AL, Woolf CJ, Lin HY: Repulsive guidance molecule (RGMa), a DRAGON homologue, is a bone morphogenetic protein co-receptor. *J Biol Chem* 2005, 280(33):29820-29827.

18. Samad TA, Rebbapragada A, Bell E, Zhang Y, Sidis Y, Jeong SJ, Campagna JA, Perusini S, Fabrizio DA, Schneyer AL et al: DRAGON, a bone morphogenetic protein co-receptor. *J Biol Chem* 2005, 280(14):14122-14129.

19. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ et al: Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat Genet* 2006, 38(5):531-539.

20. Corradini E, Babitt JL, Lin HY: The RGM/DRAGON family of BMP co-receptors. *Cytokine Growth Factor Rev* 2009, 20(5-6):389-398.

21. Zhang AS, West AP Jr, Wyman AE, Bjorkman PJ, Enns CA: Interaction of hemojuvelin with neogenin results in iron accumulation in human embryonic kidney 293 cells. *J Biol Chem* 2005, 280(40):33885-33894.

22. Zhang AS, Yang F, Wang J, Tsukamoto H, Enns CA: Hemojuvelin-neogenin interaction is required for bone morphogenetic protein-4-induced hepcidin expression. *J Biol Chem* 2009, 284(34):22580-22589.

23. Tian C, Liu J: Repulsive guidance molecules (RGMs) and neogenin in bone morphogenetic protein (BMP) signaling. *Mol Reprod Dev* 2013, 80(9):700-717.

24. Zhang R, Wu Y, Xie F, Zhong Y, Wang Y, Xu M, Feng J, Charish J, Monnier PP, Qin X: RGMa mediates reactive astrogliosis and glial scar formation through TGFbeta1/Smad2/3 signaling after stroke. *Cell Death Differ* 2018, 25(8):1503-1516.

25. Jorge EC, Ahmed MU, Bothe I, Coutinho LL, Dietrich S: RGMa and RGMb expression pattern during chicken development suggest unexpected roles for these repulsive guidance molecules in notochord formation, somitogenesis, and myogenesis. *Dev Dyn* 2012, 241(12):1886-1900.

26. Martins AF, Xavier Neto J, Azambuja A, Sereno ML, Figueira A, Campos-Junior PH, Rosario MF, Toledo CB, Silva GA, Kitten GT et al: Repulsive Guidance Molecules a, b and c Are Skeletal Muscle Proteins, and Repulsive Guidance Molecule a Promotes Cellular Hypertrophy and Is Necessary for Myotube Fusion. *Cells Tissues Organs* 2015, 200(5):326-338.

27. Sartorelli V, Fulco M: Molecular and cellular determinants of skeletal muscle atrophy and hypertrophy. *Sci STKE* 2004, 2004(244):re11.

28. Bonaldo P, Sandri M: Cellular and molecular mechanisms of muscle atrophy. *Dis Model Mech* 2013, 6(1):25-39.

29. Sartori R, Schirwis E, Blaauw B, Bortolanza S, Zhao J, Enzo E, Stantzou A, Mouisel E, Toniolo L, Ferry A et al: BMP signaling controls muscle mass. *Nat Genet* 2013, 45(11):1309-1318.

30. Otto A, Patel K: Signalling and the control of skeletal muscle size. *Exp Cell Res* 2010, 316(18):3059-3066.

31. Lyons GE, Moore R, Yahara O, Buckingham ME, Walsh FS: Expression of NCAM isoforms during skeletal myogenesis in the mouse embryo. *Dev Dyn* 1992, 194(2):94-104.

32. Nishi M, Yasue A, Nishimatu S, Nohno T, Yamaoka T, Itakura M, Moriyama K, Ohuchi H, Noji S: A missense mutant myostatin causes hyperplasia without hypertrophy in the mouse muscle. *Biochem Biophys Res Commun* 2002, 293(1):247-251.

33. Krauss RS, Cole F, Gaio U, Takaesu G, Zhang W, Kang JS: Close encounters: regulation of vertebrate skeletal myogenesis by cell-cell contact. *J Cell Sci* 2005, 118(Pt 11):2355-2362.

34. Krauss RS: Regulation of promyogenic signal transduction by cell-cell contact and adhesion. *Exp Cell Res* 2010, 316(18):3042-3049.
35. Krauss RS, Joseph GA, Goel AJ: Keep Your Friends Close: Cell-Cell Contact and Skeletal Myogenesis. Cold Spring Harb Perspect Biol 2017, 9(2).

36. McClure MJ, Ramey AN, Rashid M, Boyan BD, Schwartz Z: Integrin-alpha7 signaling regulates connexin 43, M-cadherin, and myoblast fusion. Am J Physiol Cell Physiol 2019, 316(6):C876-C887.

37. Bastos UMC, de Andrade Rosa I, Teixeira JD, Goncalves G, Costa ML, Quintas LEM, Mermelstein C: Isoproterenol induces an increase in muscle fiber size by the proliferation of Pax7-positive cells and in a mTOR-independent mechanism. Cell Biol Int 2019.

38. De Vries M, Cooper HM: Emerging roles for neogenin and its ligands in CNS development. J Neurochem 2008, 106(4):1483-1492.

39. do Carmo Costa A, Copola AGL, Carvalho ESC, Nogueira JM, Silva GAB, Jorge EC: RGMa can induce skeletal muscle cell hyperplasia via association with neogenin signalling pathway. In Vitro Cell Dev Biol Anim 2021, 57(4):415-427.

40. Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, Szczesniak MW, Gaffney DJ, Elo LL, Zhang X et al: A survey of best practices for RNA-seq data analysis. Genome Biol 2016, 17:13.

41. Wong JJ, Ritchie W, Ebner OA, Selbach M, Wong JW, Huang Y, Gao D, Pinello N, Gonzalez M, Baidya K et al: Orchestration of intron retention regulates normal granulocyte differentiation. Cell 2013, 154(3):583-595.

42. Segil N, Roberts SB, Heintz N: Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. Science 1991, 254(5039):1814-1816.

43. Bozek K, Relogio A, Kielbasa SM, Heine M, Dame C, Kramer A, Herzel H: Regulation of clock-controlled genes in mammals. PLoS One 2009, 4(3):e8482.

44. Stepchenko AG, Lyanova BM, Krylova ID, Ilyin YV, Georgieva SG, Pankratova EV: Differentiation of Monocytic Cells Is Accompanied by a Change in the Expression of the Set of Oct-1 Isoforms. Dokl Biochem Biophys 2018, 483(1):306-308.

45. Elam MB, Majumdar G, Mozhuin K, Gerling IC, Vera SR, Fish-Trotter H, Williams RW, Childress RD, Raghov R: Patients experiencing statin-induced myalgia exhibit a unique program of skeletal muscle gene expression following statin rechallenge. PLoS One 2017, 12(8):e0181308.

46. Lakich MM, Diagana TT, North DL, Whalen RG: MEF-2 and Oct-1 bind to two homologous promoter sequence elements and participate in the expression of a skeletal muscle-specific gene. J Biol Chem 1998, 273(24):15217-15226.

47. Bhavsar PK, Dellow KA, Yacoub MH, Brand NJ, Barton PJ: Identification of cis-acting DNA elements required for expression of the human cardiac troponin I gene promoter. J Mol Cell Cardiol 2000, 32(1):95-108.

48. Allen DL, Weber JN, Sycuro LK, Leinwand LA: Myocyte enhancer factor-2 and serum response factor binding elements regulate fast Myosin heavy chain transcription in vivo. J Biol Chem 2005, 280(17):17126-17134.

49. Ji GG, Shu JT, Zhang M, Ju XJ, Shan YJ, Liu YF, Tu YJ: Transcriptional regulatory region and DNA methylation analysis of TNNI1 gene promoters in Gaoyou duck skeletal muscle (Anas platyrhynchos domestica). Br Poult Sci 2019, 60(3):202-208.

50. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ et al: Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. J Clin Invest 2013, 124(1):1849-1861.

51. Hong-Brown LQ, Brown CR, Navaratnarajah M, Lang CH: Activation of AMPK/TSC2/PLD by alcohol regulates mTORC1 and mTORC2 assembly in C2C12 myocytes. Alcohol Cln Exp Res 2013, 37(11):1849-1861.

52. Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M: Mechanisms regulating skeletal muscle growth and atrophy. FEBS J 2013, 280(17):4294-4314.

53. Sartori R, Sandri M: BMPs and the muscle-bone connection. Bone 2015, 80:37-42.

54. Zhan J, Chitta RK, Harwood FC, Grosveld GC: Phosphorylation of TSC2 by PKC-delta reveals a novel signaling pathway that couples protein synthesis to mTORC1 activity. Mol Cell Biochem 2019, 456(1-2):123-134.

55. Laplante M, Sabatini DM: mTOR signaling in growth control and disease. Cell 2012, 149(2):274-293.

56. Zhang H, Cicchetti G, Onda H, Koon HB, Asrican K, Bajraszewski N, Vazquez F, Carpenter CL, Kwiatkowski DJ: Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR. J Clin Invest 2003,
The TSC1-TSC2 complex is required for proper activation of mTOR complex 2. *Mol Cell Biol* 2008, 28(12):4104-4115.

Huang J, Dibble CC, Matsuzaki M, Manning BD: **The TSC1-TSC2 complex is required for proper activation of mTOR complex 2.** *Mol Cell Biol* 2008, 28(12):4104-4115.

Phospholipase D mediates nutrient input to mammalian target of rapamycin complex 1 (mTORC1). *J Biol Chem* 2011, 286(29):25477-25486.

Potthoff MJ, Olson EN: **MEF2: a central regulator of diverse developmental programs.** *Development* 2007, 134(23):4131-4140.

Liu N, Nelson BR, Bezprozvannaya S, Shelton JM, Richardson JA, Bassel-Duby R, Olson EN: Requirement of MEF2A, C, and D for skeletal muscle regeneration. *Proc Natl Acad Sci U S A* 2014, 111(11):4109-4114.

Schiano S, Dyar KA, Calabria E: **Skeletal muscle mass is controlled by the MRF4-MEF2 axis.** *Curr Opin Clin Nutr Metab Care* 2018, 21(3):164-167.

Xu L, Salloum D, Medlin PS, Saqcena M, Yellen P, Perrella B, Foster DA: Phospholipase D mediates nutrient input to mammalian target of rapamycin complex 1 (mTORC1). *J Biol Chem* 2011, 286(29):25477-25486.

Potthoff MJ, Olson EN: **MEF2: a central regulator of diverse developmental programs.** *Development* 2007, 134(23):4131-4140.

Xu L, Salloum D, Medlin PS, Saqcena M, Yellen P, Perrella B, Foster DA: Phospholipase D mediates nutrient input to mammalian target of rapamycin complex 1 (mTORC1). *J Biol Chem* 2011, 286(29):25477-25486.

Potthoff MJ, Olson EN: **MEF2: a central regulator of diverse developmental programs.** *Development* 2007, 134(23):4131-4140.

Xu L, Salloum D, Medlin PS, Saqcena M, Yellen P, Perrella B, Foster DA: Phospholipase D mediates nutrient input to mammalian target of rapamycin complex 1 (mTORC1). *J Biol Chem* 2011, 286(29):25477-25486.
78. Tomczak KK, Marinescu VD, Ramoni MF, Sanoudou D, Montanaro F, Han M, Kunkel LM, Kohane IS, Beggs AH: Expression profiling and identification of novel genes involved in myogenic differentiation. *FASEB J* 2004, 18(2):403-405.

79. Lee EJ, Malik A, Pokharel S, Ahmad S, Mir BA, Cho KH, Kim J, Kong JC, Lee DM, Chung KY *et al.* Identification of genes differentially expressed in myogenin knock-down bovine muscle satellite cells during differentiation through RNA sequencing analysis. *PLoS One* 2014, 9(3):e92447.

80. Shang N, Lee JTY, Huang T, Wang C, Lee TL, Mok SC, Zhao H, Chan WY: Disabled-2: a positive regulator of the early differentiation of myoblasts. *Cell Tissue Res* 2020, 381(3):493-508.

81. Bannister RA: Bridging the myoplasmic gap: recent developments in skeletal muscle excitation-contraction coupling. *J Muscle Res Cell Motil* 2007, 28(4-5):275-283.

82. Nakada T, Kashihara T, Komatsu M, Kojima K, Takeshita T, Yamada M: Physical interaction of junctophilin and the CaV1.1 C terminus is crucial for skeletal muscle contraction. *Proc Natl Acad Sci U S A* 2018, 115(17):4507-4512.

83. Park JW, Lee JH, Kim SW, Han JS, Kang KS, Kim SJ, Park TS: Muscle differentiation induced up-regulation of calcium-related gene expression in quail myoblasts. *Asian-Australas J Anim Sci* 2018, 31(9):1507-1515.

84. Tajhya RB, Hu X, Tanner MR, Huq R, Kongchan N, Neilson JR, Rodney GG, Horrigan FT, Timchenko LT, Beeton C: Functional KCa1.1 channels are crucial for regulating the proliferation, migration and differentiation of human primary skeletal myoblasts. *Cell Death Dis* 2016, 7(10):e2426.

85. Liu JH, Konig S, Michel M, Amaudeau S, Fischer-Lougheed J, Bader CR, Bernheim L: Acceleration of human myoblast fusion by depolarization: graded Ca2+ signals involved. *Development* 2003, 130(15):3437-3446.

86. Porter GA, Jr., Makuck RF, Rivkees SA: Reduction in intracellular calcium levels inhibits myoblast differentiation. *J Biol Chem* 2002, 277(32):28942-28947.

87. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C: Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods* 2017, 14(4):417-419.

88. Love MI, Huber W, Anders S: Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014, 15(12):550.

89. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, Fridman WH, Pages F, Trajanoski Z, Galon J: ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* 2009, 25(8):1091-1093.

90. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T: Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003, 13(11):2498-2504.

91. Pfaffl MW, Horgan GW, Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002, 30(9):e36.

**Figures**
Figure 1

Quality and transcriptomic profile of RGMa-treated myoblasts during myogenic differentiation. (A) Pearson Correlation Coefficient (PCC) analysis of normalised read-counts denoted a high internal consistency and reproducibility of treated and control replicates. (B) MA plot analysis showing the RNA-seq profile of the log2 (fold change) distributions of all DETs in the average of normalised counts. Each point represents one transcript. Those dots marked in blue were detected as differentially expressed at a 5% FDR with log2(FC) > 0 (upregulated) and log2(FC) < 0 (downregulated) after RGMa treatment. Transcripts with similar expression levels are represented around the horizontal line (y = 0). Dots that are outside the window are plotted as triangles. (C) Heatmap analysis of DET with muscle-associated terms ('cellular component,' 'biological process,' and 'molecular function') of Gene Ontology (GO). Transcripts with the lowest expression values are marked in red, median expression values in black, and the highest expression values in green.
Figure 2

RNA biotypes modulated by RGMa treatment. RGMa could modulate the differential expression of 13 RNA biotypes, classified in six RNA categories according to Ensembl (https://m.ensembl.org/info/genome/genebuild/biotypes.html): (1) protein coding, (2) processed transcripts (IncRNA: antisense, bidirection-promoter-IncRNA, lincRNA, retained intron and ncRNA: snRNA and Mt-rRNA), (3) nonsense mediated decay, (4) pseudogenes (processed-pseudogenes, transcribed-processed-pseudogene, and unprocessed-pseudogene), and (5) Tec (to be experimentally confirmed).
Figure 3

Functional analysis of the non-protein coding RNA differentially regulated by RGMa treatment. For this analysis, we considered upregulated DETs that do not encode proteins. Pie analysis of the GO enrichment, showing the most frequent terms, including cellular component, biological process, molecular function, and immune system process, and KEGG GO terms that were (A) upregulated and (B) downregulated. The right-sided hypergeometric test was used in statistical inference, and the Benjamini-Hochberg method was applied for a p-value correlation (p < 0.05). The analysis was conducted using the plugin ClueGO (v.2.5.4) for Cytoscape (v3.7.1).
Figure 4

Functional analysis of the protein coding RNA upregulated by RGMa. For this analysis, we considered the DETs that encode proteins that were found to be upregulated (FC > 1) by the treatment with RGMa, compared to the control. (A-C) Pie chart analysis of the three GO categories used to classify the upregulated protein coding transcripts. The right-sided hypergeometric test was used in statistical inference, and the Benjamini-Hochberg method was applied for a p-value correlation (p < 0.0001). The analysis was conducted using the plugin ClueGO (v.2.5.4) for Cytoscape (v3.7.1).
Figure 5

Functional analysis of the protein coding RNA downregulated by RGMa. For this analysis, we considered the DETs that encode proteins and were found to be downregulated (FC < 1) in the RGMa treated group, compared to the control one. (A-C) Pie chart analysis of the three GO categories for downregulated DETs. (D) Functionally grouped network of enriched categories for expressed transcripts, annotated for ‘biological process,’ ‘cellular component,’ and ‘molecular function’ GO terms. The right-sided hypergeometric test was used in statistical inference, and the Benjamini-Hochberg method was applied for a p-value correlation (p<0.001). The analysis was conducted using the plugin ClueGO (v.2.5.4) for Cytoscape (v3.7.1).

Figure 6

Muscle-related enriched terms from the functional analysis of all DETs in response to rcRGMa. Functionally grouped network of enriched categories for all types of DET annotated as ‘cellular component,’ ‘biological process,’ and ‘molecular function’ GO terms. For this analysis, we considered the DETs that were upregulated (A) and downregulated (B) in the RGMa-treated group, compared to the control group. Only the most significant term in the group was labelled. GO terms are represented as nodes,
and the node size represents the term enrichment significance. The edges connecting the nodes are based on the Kappa statistic (Kappa Score Threshold of 0.4), which measures the overlap of shared genes between terms. The right-sided hypergeometric test was used in statistical inference, and the Benjamini-Hochberg method was applied for a p-value correlation (p < 0.05). The analysis was conducted using the plugin ClueGO (v.2.5.4) for Cytoscape (v3.7.1).

**Figure 7**

Validation of the RNA-seq expression profiles by qPCR. A subset of twelve DETs that were upregulated and downregulated by RGMa treatment during muscle differentiation were used to validate the obtained RNA-seq expression data. Transcripts were selected by their expression and their known association with muscle hyperplasic or hypertrophic phenotypes. Expression patterns indicate agreement between the two methods and *, significance of p-adj < 0.05.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xlsx