



RESEARCH NOTE

REVISED **Strigolactone GR24 upregulates target genes of the cytoprotective transcription factor Nrf2 in skeletal muscle [version 2; peer review: 2 approved]**

Previously titled: Strigolactone GR24 upregulates Nrf2 target genes and may protect against oxidative stress in skeletal muscle

Shalem Raju Modi , Tarja Kokkola 

Department of Internal Medicine, Institute of Clinical Medicine, University of Eastern Finland, Kuopio, 70210, Finland

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Abstract







GR24 is a synthetic strigolactone analog, demonstrated to regulate the development of plants and arbuscular mycorrhizal fungi. GR24 possesses anti-cancer and anti-apoptotic properties, enhances insulin sensitivity and mitochondrial biogenesis in skeletal myotubes, inhibits adipogenesis, decreases inflammation in adipocytes and macrophages and downregulates the expression of hepatic gluconeogenic enzymes. Transcription factor Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) is a master regulator of antioxidant response, regulating a multitude of genes involved in cellular stress responses and anti-inflammatory pathways, thus maintaining cellular redox homeostasis. Nrf2 activation reduces the deleterious effects of mitochondrial toxins and has multiple roles in promoting mitochondrial function and dynamics. We studied the role of GR24 on gene expression in rat L6 skeletal muscle cells which were differentiated into myotubes. The myotubes were treated with GR24 and analyzed by microarray gene expression profiling. GR24 upregulated the cytoprotective transcription factor Nrf2 and its target genes, activating antioxidant defences, suggesting that GR24 may protect skeletal muscle from the toxic effects of oxidative stress.

Keywords

Strigolactone, GR24, Nrf2, Oxidative stress, Microarray

Open Peer Review

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- David A. Hood**, York University, Toronto, Canada
- Tugba Boyunegmez Tumer**, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Shalem Raju Modi (shalem.modi@uef.fi)

Author roles: **Modi SR:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Kokkola T:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

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REVISED Amendments from Version 1

In agreement with the valid comments of the reviewers, we have changed the title and conclusion.

See referee reports

Introduction

Strigolactones are carotenoid-derived phytohormones with endogenous roles in regulating plant growth and exogenous roles in establishing symbiosis of host plant with arbuscular mycorrhizal fungi¹. Strigolactones induce beneficial effects in mycorrhiza, such as mitochondrial biogenesis and ATP production²⁻⁴. The anti-cancer properties⁵⁻⁸ and anti-inflammatory potential⁹ of strigolactones have recently been investigated in mammalian cells. The positive effects of strigolactone analog GR24 in enhancing insulin sensitivity, mitochondrial function and inhibiting adipogenesis and inflammation in insulin-sensitive cells have also been demonstrated^{10,11}.

Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) activates gene transcription by binding to the antioxidant response element (ARE) in the promoters of its target genes. It regulates multiple biological functions ranging from cellular redox metabolism, detoxification, heme, lipid and glucose metabolism, NADPH generation, autophagy, apoptosis, xenobiotic stress response to inflammation by interacting with its target genes, regulating an extensive antioxidant protein network. Nrf2 is associated with disease pathologies like cancer¹², hepatotoxicity¹³, cardiovascular disease¹⁴ and neurodegenerative diseases¹⁵.

Mitochondria are the major sites of reactive oxygen species (ROS) production and also the targets of their toxic effects. Mitochondrial dysfunction has been associated with the development of insulin resistance, and diabetes is known to induce oxidative stress through the overproduction of ROS and ROS-induced DNA damage¹⁶. Nrf2 activation defends against mitochondrial toxins and ROS and affects mitochondrial function by regulating mitochondrial biogenesis, mitochondrial fatty acid oxidation, respiration, ATP production and mitochondrial dynamics¹⁷. With its versatile protective mechanism against oxidative stress, pancreatic β -cell apoptosis and insulin resistance, Nrf2 has become a promising therapeutic target for the treatment of type 2 diabetes¹⁸.

We have demonstrated that GR24 ameliorates insulin sensitivity, stimulates mitochondrial biogenesis and ATP production and upregulates genes regulating mitochondrial function in L6 myotubes¹⁰. Very recently, the efficacy of GR24 in promoting cytoprotective responses via Nrf2 activation was reported in hepatic and macrophage cell lines¹⁹. This work reports a transcriptomic study revealing the potential beneficial effects of GR24 in upregulating Nrf2 and its target genes involved in detoxification, carbohydrate and lipid metabolism, heme metabolism, NADPH regeneration and oxidative stress in L6 myotubes, thus contributing to metabolic homeostasis.

Methods

Results from the methods described in this study have been published previously¹⁰, although the analyses described here are published for the first time.

Cell culture and differentiation

Rat L6 myoblasts (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM 4.5 g/l glucose, Lonza, Basel, Switzerland) supplemented with 10% FBS (Hyclone, Pasching, Austria), 2 mM L-glutamine (Lonza) and 1% penicillin/streptomycin (Lonza) at +37°C in a humidified atmosphere of 5% CO₂. The cells were seeded in multiwell plates at 2 × 10⁴ cells/cm² one day before starting the differentiation. Myoblasts were differentiated into myotubes by switching into α -MEM media (Gibco, Paisley, UK) supplemented with 2% horse serum (Gibco) and 1% penicillin/streptomycin. GR24 (3E,3aR,8bS)-3-[[[(2S)-4-methyl-5-oxo-2H-furan-2-yl]oxymethylidene]-4,8b-dihydro-3aH-indeno[1,2-b]furan-2-one, Chiralix, Nijmegen, Netherlands) was dissolved in DMSO. The control samples had equivalent DMSO concentration.

Microarray sample preparation

L6 myotubes were treated with 60 μ M GR24 at 7 days of differentiation for 24 h in three independent experiments, resulting in three replicate microarrays in each treatment group. Total RNAs were extracted with RNeasy Mini kit (Qiagen, Hilden, Germany) and RNA quality was assessed with the Agilent Bioanalyzer 2100 (Agilent Technologies, Espoo, Finland). Total RNA (200 ng) was converted to cDNA with Agilent AffinityScript RNase Block, labelled according to manufacturer's instructions and purified using RNeasy mini spin columns (Qiagen). RNA Spike-In Kit (Exiqon, Vedbaek, Denmark) was used to monitor the success of labelling. Samples were then mixed with blocking agent, fragmentation and hybridization buffer, and were hybridized to Agilent SurePrint G3 Rat GE 8 × 60 K Microarrays for 17 h at 65°C before washing and scanning with Agilent Scanner G2505C using manufacturer's protocols. Agilent Feature Extraction software was used to extract data from raw microarray image files¹⁰.

Microarray data processing

The data was processed with **limma** package (version 3.28.21) of the **Bioconductor** software²⁰. Microarray data are MIAME compliant. Differentially expressed transcripts were analysed by Bayes moderated t-statistics followed by the Benjamini-Hochberg correction method to control false discovery rate (FDR) with significance threshold set at $p < 0.05$ ^{20,21}.

Results and discussion

The top 5 GR24-upregulated genes (glutathione S-transferase alpha 1, metallothionein 1M, heme oxygenase 1, glutathione S-transferase alpha 2, and sequestosome 1) were found to be known targets of Nrf2 (Table 1). As the prototypical Nrf2 target gene NAD(P)H quinone dehydrogenase 1 (*Nqo1*) was also found high on the list, it was compelling to look into the extensive list of Nrf2 target genes. We found 56 known Nrf2 target genes²², including Nrf2 itself, to be upregulated by GR24 treatment

Table 1. Selected upregulated Nrf2 target genes in L6 myotubes after 24 h treatment with 60 µM GR24.

Gene symbol	Description	Accession	logFC	Fold change	Adjusted P value
Detoxication: Phase I drug oxidation, reduction and hydrolysis					
<i>Adh7</i>	Alcohol dehydrogenase 7	NM_134329	1.604	3.04	1.746E-09
<i>Akr1b1</i>	Aldo-keto reductase family 1 member B1	NM_012498	1.831	3.56	7.986E-09
<i>Akr1b8</i>	Aldo-keto reductase family 1. member B8	NM_173136	2.503	5.67	1.254E-10
<i>Akr1cl</i>	Aldo-keto reductase family 1. member C-like	NM_001109900	0.685	1.61	7.675E-06
<i>Cbr1</i>	Carbonyl reductase 1	NM_019170	1.060	2.08	3.055E-08
<i>Nqo1</i>	NAD(P)H quinone dehydrogenase 1	NM_017000	3.144	8.84	1.602E-10
<i>Ptgr1</i>	Prostaglandin reductase 1	NM_138863	3.233	9.40	1.196E-09
Detoxication: Phase II drug conjugation					
<i>Gsta1</i>	Glutathione S-transferase alpha 1	NM_031509	4.610	24.42	3.458E-11
<i>Gsta2</i>	Glutathione S-transferase alpha 2	NM_001010921	3.676	12.78	1.348E-09
<i>Gstm1</i>	Glutathione S-transferase mu 1	NM_017014	0.707	1.63	7.461E-06
<i>Gstp1</i>	Glutathione S-transferase pi 1	NM_012577	2.498	5.65	3.862E-10
<i>Mgst1</i>	Microsomal glutathione S-transferase 1	NM_134349	2.327	5.02	1.638E-09
<i>Ugt1a2</i>	UDP glucuronosyltransferase 1 family. polypeptide A2	NM_001039691	0.169	1.12	3.123E-02
Detoxication: Phase III drug transport					
<i>Abcb6</i>	ATP binding cassette subfamily B member 6	NM_080582	0.194	1.14	6.514E-03
<i>Abcc1</i>	ATP binding cassette subfamily C member 1	NM_022281	1.420	2.68	2.985E-07
<i>Abcc2</i>	ATP binding cassette subfamily C member 2	NM_012833	0.653	1.57	2.263E-03
<i>Abcc4</i>	ATP binding cassette subfamily C member 4	NM_133411	1.367	2.58	4.359E-07
<i>Abcc5</i>	ATP binding cassette subfamily C member 5	NM_053924	0.807	1.75	4.986E-06
Antioxidant systems					
<i>Cat</i>	Catalase	NM_012520	2.205	4.61	7.440E-09
<i>Gclc</i>	Glutamate-cysteine ligase, catalytic subunit	NM_012815	1.231	2.35	2.616E-07
<i>Ggt1</i>	Gamma-glutamyltransferase 1	NM_053840	2.590	6.02	6.774E-09
<i>Glx</i>	Glutaredoxin	NM_022278	0.717	1.64	9.108E-05
<i>Gls</i>	Glutaminase	NM_012569	1.782	3.44	2.165E-09
<i>Gpx4</i>	Glutathione peroxidase 4	NM_001039849	0.451	1.37	1.766E-05
<i>Prdx1</i>	Peroxiredoxin 1	NM_057114	1.361	2.57	1.274E-06
<i>Prdx6</i>	Peroxiredoxin 6	NM_053576	1.291	2.45	9.933E-06
<i>Slc6a9</i>	Solute carrier family 6 member 9 (glycine transporter)	NM_053818	2.283	4.87	9.548E-10
<i>Slc7a11</i>	Solute carrier family 7 member 11 (cystine/glutamate transporter)	NM_001107673	0.870	1.83	5.449E-06
<i>Sod1</i>	Superoxide dismutase 1	NM_017050	0.939	1.92	2.031E-06
<i>Srxn1</i>	Sulfiredoxin 1	NM_001047858	2.330	5.03	7.817E-10
<i>Txn1</i>	Thioredoxin	NM_053800	1.712	3.28	4.093E-09
<i>Txnrd1</i>	Thioredoxin reductase 1	NM_031614	2.193	4.57	1.765E-08

Gene symbol	Description	Accession	logFC	Fold change	Adjusted P value
Carbohydrate metabolism and NADPH regeneration					
<i>G6pd</i>	Glucose-6-phosphate dehydrogenase	NM_017006	2.184	4.54	7.894E-09
<i>Idh1</i>	Isocitrate dehydrogenase 1	NM_031510	1.096	2.14	4.304E-08
<i>Me1</i>	Malic enzyme 1	NM_012600	0.860	1.81	8.162E-08
<i>Pgd</i>	Phosphogluconate dehydrogenase	NM_001305435	1.920	3.79	1.503E-09
<i>Taldo1</i>	Transaldolase 1	NM_031811	1.150	2.22	2.143E-07
<i>Tkt</i>	Transketolase	NM_022592	0.989	1.99	2.321E-06
<i>Ugdh</i>	UDP-glucose 6-dehydrogenase	NM_031325	0.623	1.54	2.451E-05
Lipid metabolism					
<i>Acot7</i>	Acyl-coa thioesterase 7	NM_001146061	0.852	1.80	6.273E-07
<i>Acox1</i>	Acyl-coa oxidase 1	NM_017340	0.706	1.63	1.897E-05
<i>Ces1a</i>	Carboxylesterase 1A	NM_001190375	1.132	2.19	7.021E-05
<i>Pnpla2</i>	Patatin-like phospholipase domain-containing 2	NM_001108509	0.424	1.34	7.480E-05
Heme and iron metabolism					
<i>Blvra</i>	Biliverdin reductase A	NM_053850	0.890	1.85	1.973E-06
<i>Blvrb</i>	Biliverdin reductase B	NM_001106236	1.497	2.82	5.723E-09
<i>Fech</i>	Ferrochelatase	NM_001108434	1.431	2.70	5.570E-09
<i>Fth1</i>	Ferritin heavy chain 1	NM_012848	0.877	1.84	4.826E-07
<i>Ftl1</i>	Ferritin light chain 1	NM_022500	1.017	2.02	3.213E-07
<i>Hmox1</i>	Heme oxygenase 1	NM_012580	3.959	15.55	1.136E-10
Transcription factors and associated proteins					
<i>Ahr</i>	Aryl hydrocarbon receptor	NM_013149	0.867	1.82	1.519E-06
<i>Cebpb</i>	CCAAT/enhancer binding protein beta	NM_001301715	0.959	1.94	1.102E-06
<i>Mafg</i>	Mafg protein	NM_022386	0.718	1.64	2.006E-05
<i>Mt1m</i>	Metallothionein 1M	NM_001137564	4.009	16.10	7.970E-10
<i>Nfe2l2</i>	Nuclear factor, erythroid 2-like 2 (Nrf2)	NM_031789	0.659	1.58	3.116E-05
<i>Rxra</i>	Retinoid X receptor alpha	NM_012805	0.788	1.73	1.677E-05
<i>Sqstm1</i>	Sequestosome 1	NM_181550	3.576	11.93	3.458E-11
Ubiquitin ligase substrate adaptor					
<i>Keap1</i>	Kelch-like ECH-associated protein 1	NM_057152	1.269	2.41	3.765E-08

(Table 1). Prior to our study, the effects of strigolactones treatment on the mammalian cell transcriptome have only been investigated in human osteosarcoma cells, where strigolactone analogs mainly upregulated the heat shock stress proteins and downregulated the cell cycle⁵.

Activation of Nrf2 signaling is known to have beneficial effects in cancer, metabolic syndrome, obesity, nephropathy, retinopathy, neuropathy, and β -cell protection. Nrf2 regulates the transcription of genes involved in antioxidant, detoxification

and metabolic processes²³. In our study, GR24 enhanced the expression of the Nrf2-dependent antioxidant genes *Nqo1* and heme oxygenase 1, which are known to block inflammatory pathways²⁴. GR24 has recently been shown to alleviate inflammation and upregulate these two NRF2 target genes in hepatic and macrophage cells¹⁹.

Glutathione and associated enzymes form an important antioxidant defence system. Treatment with GR24 was found to increase root glutathione content in plants²⁵, but there are no previous

reports on the effects of strigolactones on the glutathione system in mammalian cells. Our results show that GR24 treatment upregulated glutamate-cysteine ligase, the rate-limiting enzyme in glutathione synthesis (Table 1). Many components of the glutathione system are upregulated by Nrf2, and the elevations in the expression of glutaredoxin, glutathione peroxidase 4 and several glutathione S-transferases in GR24-treated cells were evident in our results (Table 1).

Conclusions

GR24 upregulated the cytoprotective transcription factor Nrf2 and its target genes, which are involved in detoxification, antioxidant systems, carbohydrate and lipid metabolism, heme and iron metabolism, NADPH regeneration and regulation of transcription in L6 myotubes. Future research aimed to elucidate the effects of GR24 in the oxidative stress mechanisms in skeletal muscle at the protein level may provide supporting evidence about the therapeutic potential of this compound.

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Data availability

Microarray raw data have been deposited in the NCBI Gene Expression Omnibus (GEO), accession number GSE90833: <https://identifiers.org/geo/GSE90833>.

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The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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David A. Hood

Muscle Health Research Centre, School of Kinesiology and Health Science, York University, Toronto, ON, Canada

I have reviewed the responses and am satisfied with the responses. I have no further comments to add.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 14 November 2018

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Tugba Boyunegmez Tumer

Department of Molecular Biology and Genetics, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

Authors have carried out satisfactory changes in the revised MS. In this form, it can be acceptable for indexing.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 31 October 2018

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**Tugba Boyunegmez Tumer**

Department of Molecular Biology and Genetics, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

In current research note, a microarray study was carried out to analyze the effect of GR24, a synthetic strigolactone analog, on the gene expression profile of rat L6 myotubes. 60 µM of GR24 was used to treat L6 myotubes at 7th day of differentiation for 24 h in three independent experiments. Microarray data was processed and differentially expressed transcripts were analyzed. Upregulated genes were partly presented in Table 1. Accordingly, GR24 upregulated the cytoprotective transcription factor Nrf2 and its several target genes involved in antioxidant defense and phase II detoxification processes. The fold values especially for *GSTa1* and *a2*, *NQO1*, *GGT1*, *Hmox1*, *Mtm1m*, *Sqstm1* were considerably and significantly high (above 6 fold). Authors previously showed that GR24 (60 µM) ameliorates insulin sensitivity, stimulates mitochondrial biogenesis and ATP production and upregulates genes regulating mitochondrial function in L6 myotubes (Modi et al., 2017¹).

Also, in this previous report it was shown that 9838 transcripts were upregulated and 6315 transcripts were downregulated in L6 myotubes as a result of 60 µM GR24 treatment. Up and down regulated transcripts involved in insulin signaling and mitochondrial function in L6 myotubes have been presented in this previous paper (Modi et al., 2017¹). In the present research note, authors represented up regulated transcripts involved in oxidative stress and cytoprotective signaling when myotubes were treated with 60 µM GR24. Since the work can be classified as original and the results are remarkable, data deserves to be published in F1000Research as research note. However, the data is limited to reach the conclusion suggesting that GR24 may protect skeletal muscle from the toxic effects of oxidative stress and thus contributing to metabolic homeostasis. In the scope of report, there is no any biological assay or disease modelling evaluating the beneficial effects of GR24 on myotubes. Furthermore, gene expression profiling does not always reflect the changes on the protein level. Regarding these facts, title also should not contain the assumption suggesting that GR24 may protect against oxidative stress in skeletal muscle.

In my opinion, both conclusion and title need revision and if possible it is better to represent additional data and interpretation in terms of down regulated genes in specified signaling pathways. Also could you please add an explanation about why you chose 60 µM concentration?

References

1. Modi S, Yaluri N, Kokkola T, Laakso M: Plant-derived compounds strigolactone GR24 and pinosylvin activate SIRT1 and enhance glucose uptake in rat skeletal muscle cells. *Sci Rep.* 2017; 7 (1): 17606 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 07 Nov 2018

SHALEM RAJU MODI, University of Eastern Finland, Kuopio, Finland

We thank the referee for kindly evaluating our research note and for the valid comments and suggestions. We agree with the comments and hence, we changed the discussion part and also the title of the manuscript.

Also could you please add an explanation about why you chose 60 μM concentration?

We initially tested GR24 at various concentrations from 20 μM – 100 μM . We observed that at 60 μM concentration, GR24 significantly upregulated SIRT1 protein expression and enhanced glucose uptake in skeletal muscle cells (Sci Rep. 2017; 7(1): 17606.). Moreover, longer exposure to 100 μM GR24 had a small but significant cytotoxic effect. Hence, we have chosen 60 μM to be an ideal concentration for all our experiments.

Competing Interests: No competing interests were disclosed.

Reviewer Report 02 October 2018

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**David A. Hood**

Muscle Health Research Centre, School of Kinesiology and Health Science, York University, Toronto, ON, Canada

Interesting investigation of the effect of a plant-based compound to change gene expression in muscle cells, however the study is very limited in scope.

1. All reported data show increases in mRNA. Are there no significant decreases that are noteworthy?
2. Have any changes in mRNA using microarrays been verified with Q-PCR? This is standard practice;
3. Could a few protein blots be included to strengthen the data and take the interpretation to the protein level?
4. The title assumes that the mRNA changes lead to protein changes which would impact oxidative stress. The title needs to be re-worded unless actual measures of oxidative stress are performed. This would be relatively easy with an oxi-blot, or ROS measure in cells.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 19 Oct 2018

SHALEM RAJU MODI, University of Eastern Finland, Kuopio, Finland

We thank the referee for carefully evaluating our research note. He has correctly identified that the study has its limitations. As we have been engaged in other studies and have not been able to dedicate more time for continuing on this track, we decided to submit our microarray analyses in the current format. This Research Note format facilitates publishing small studies, such as our study with only a single table.

1. All reported data show increases in mRNA. Are there no significant decreases that are noteworthy?

In the current short publication, we do not report all significant findings. A total of 16,153 transcripts were differentially regulated (9,838 up- and 6,315 downregulated) to the GR24 treatment. Some of the differentially regulated transcripts have been described in our previous publication (Modi S, Yaluri N, Kokkola T, et al.: Plant-derived compounds strigolactone GR24 and pinosylvin activate SIRT1 and enhance glucose uptake in rat skeletal muscle cells. *Sci Rep.* 2017; 7(1): 17606.). Nevertheless, the most interesting finding from the microarray experiments was the strong upregulation of Nrf2 target transcripts by GR24 treatment.

2. Have any changes in mRNA using microarrays been verified with Q-PCR? This is standard practice;

3. Could a few protein blots be included to strengthen the data and take the interpretation to the protein level?

4. The title assumes that the mRNA changes lead to protein changes which would impact oxidative stress. The title needs to be re-worded unless actual measures of oxidative stress are performed. this would be relatively easy with an oxi-blot, or ROS measure in cells.

These are very valid questions. The suggested qPCR analyses, protein blots and measures of oxidative stress would be essential next steps to verify the changes in gene expression. For that reason, we published our preliminary analyses as a Research Note, as additional analyses would have been required in order to produce a complete full-length article. When drafting the title, we avoided making strong claims on the effects on oxidative stress. After reading the suggestions by the referee, we have reconsidered the title, and will submit a new version of the article with a title "Strigolactone GR24 upregulates target genes of the cytoprotective transcription factor Nrf2 in skeletal muscle".

Competing Interests: No competing interests were disclosed.

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