Author/s:
Hagg, A;Colgan, TD;Thomson, RE;Qian, H;Lynch, GS;Gregorevic, P

Title:
Using AAV vectors expressing the β 2 -adrenoceptor or associated Gα proteins to modulate skeletal muscle mass and muscle fibre size

Date:
2016-03-14

Citation:
Hagg, A., Colgan, T. D., Thomson, R. E., Qian, H., Lynch, G. S. & Gregorevic, P. (2016). Using AAV vectors expressing the β 2 -adrenoceptor or associated Gα proteins to modulate skeletal muscle mass and muscle fibre size. Scientific Reports, 6 (1), https://doi.org/10.1038/srep23042.

Persistent Link:
https://hdl.handle.net/11343/240784

SCIENTIFIC REPORTS

OPEN

Received: 02 October 2015
Accepted: 25 February 2016
Published: 14 March 2016

Using AAV vectors expressing the β_2-adrenoceptor or associated Gα proteins to modulate skeletal muscle mass and muscle fibre size

Adam Hagg[1,2], Timothy D. Colgan[1,2], Rachel E. Thomson[1], Hongwei Qian[1], Gordon S. Lynch[2] & Paul Gregorevic[1,2,3,4]

Anabolic β_2-adrenoceptor (β_2-AR) agonists have been proposed as therapeutics for treating muscle wasting but concerns regarding possible off-target effects have hampered their use. We investigated whether β_2-AR-mediated signalling could be modulated in skeletal muscle *via* gene delivery to the target tissue, thereby avoiding the risks of β_2-AR agonists. In mice, intramuscular administration of a recombinant adeno-associated virus-based vector (rAAV vector) expressing the β_2-AR increased muscle mass by >20% within 4 weeks. This hypertrophic response was comparable to that of 4 weeks' treatment with the β_2-AR agonist formoterol, and was not ablated by mTOR inhibition. Increasing expression of inhibitory (Gαi2) and stimulatory (GαsL) G-protein subunits produced minor atrophic and hypertrophic changes in muscle mass, respectively. Furthermore, Gαi2 over-expression prevented AAV:β_2-AR mediated hypertrophy. Introduction of the non-muscle Gαs isoform, GαsXL elicited hypertrophy comparable to that achieved by AAV:β_2-AR. Moreover, GαsXL gene delivery was found to be capable of inducing hypertrophy in the muscles of mice lacking functional β_1- and β_2-ARs. These findings demonstrate that gene therapy-based interventions targeting the β_2-AR pathway can promote skeletal muscle hypertrophy independent of ligand administration, and highlight novel methods for potentially modulating muscle mass in settings of disease.

Over 800 G-protein coupled receptor (GPCR) variants are encoded by the human genome[1]. As transmembrane receptors, the GPCRs represent the target of nearly one-third of all pharmaceuticals developed to date[2]. One of the best characterized GPCRs in skeletal muscle is the β_2-adrenoceptor (β_2-AR)[3]. *In vivo*, endogenous catecholamines such as adrenaline activate skeletal muscle β_2-ARs to promote receptor interaction with stimulatory (Gαs) and inhibitory (Gαi) G-proteins[4]. The activation of these intracellular effectors differentially regulates adenylyl cyclase (AC) activity and subsequent cAMP accumulation, which impacts on several cellular mechanisms that influence the muscle phenotype[5]. Chronic stimulation of skeletal muscle β_2-ARs through administration of β_2-AR agonists such as clenbuterol, fenoterol and formoterol has well-characterized anabolic consequences, resulting in increased muscle mass and force-producing capacity[6,7]. Anabolism of skeletal muscle following β_2-AR agonist administration has been associated with increased protein synthesis *via* stimulation of the Akt-mTOR-S6 kinase signalling axis[8,9]. However, β_2-AR agonist administration can also attenuate protein degradation by repressing transcription of the muscle-specific E3 ubiquitin ligases Murf1 and Atrogin-1, and Ca^{2+}-dependent proteases[10–12].

Because sustained stimulation of β_2-AR in skeletal muscle supports anabolic and anti-catabolic processes, synthetic β_2-AR agonists have been investigated as potential therapeutics to combat the loss of muscle mass and force-producing capacity associated with conditions such as neurogenic muscle atrophy[9,13,14], muscular dystrophy[15–17], sarcopenia[6,18], and cancer cachexia[7,19,20]. However, the expression of β_2-ARs in other cell types has prompted concerns about the risks of off-target effects arising from long-term systemic administration of β_2-AR agonists. Consequently, clinical application of these compounds for muscle wasting has remained limited. We investigated whether stimulation of β_2-AR signalling that promotes skeletal muscle hypertrophy might be

[1]Baker IDI Heart and Diabetes Institute, Melbourne, 3004, Australia. [2]Dept. of Physiology, The University of Melbourne, Melbourne, 3010, Australia. [3]Dept. of Biochemistry and Molecular Biology, Monash University, Clayton, 3800, Australia. [4]Department of Neurology, University of Washington School of Medicine, Seattle, 98195, USA. Correspondence and requests for materials should be addressed to P.G. (email: paul.gregorevic@bakeridi.edu.au)

achievable by means that circumvent the potential off-target effects of β_2-AR agonists. Specifically, we hypothesised that administering gene therapy-based interventions to alter the expression of β_2-AR pathway components could promote skeletal muscle growth independent of β_2-AR agonist administration. This rationale was based on the emerging development of recombinant adeno-associated virus-based vectors (rAAV vectors) as tools for therapeutic gene delivery, owing to their propensity for achieving efficacious and targeted delivery of transgenes to the skeletal muscles of mammals[21,22], including humans[23,24], that can sustain transgene expression for over a decade following a single treatment[24].

Our studies identified that β_2-AR gene delivery using rAAV vectors can promote skeletal muscle hypertrophy in mice without administration of synthetic β_2-AR agonists. Additionally, we observed that increasing the expression of specific G-protein subunits could exert hypertrophic and atrophic effects in skeletal muscle independent of ligand administration. These studies introduce targeted gene delivery as a new strategy for manipulating the β_2-AR signalling pathway without administering β_2-AR agonists, to promote skeletal muscle hypertrophy.

Results

β_2-AR gene delivery promotes skeletal muscle hypertrophy and protein synthesis. To determine the effects of increasing β_2-AR abundance in muscle fibres, we used adeno-associated virus-based vectors encoding the β_2-AR (AAV:β_2-AR) or a gene-less cassette (control) to transduce the tibialis anterior (TA) hindlimb muscles of male eight-week-old C57Bl/6 mice. Optimisation of vector doses established that injection of muscles with 1×10^{10} AAV:β_2-AR vector genomes (vg) produced a 22% increase in muscle mass within 28 days of vector administration, which was maintained for at least 84 days after vector delivery (the longest time point examined) (Fig. 1a). Cross-sections of muscles immunolabelled for β_2-AR and laminin confirmed widespread expression of β_2-AR on the sarcolemma of transduced muscle fibres (Fig. 1b), and an increase in the diameter of muscle fibres in treated muscles (Fig. 1c). Muscles administered AAV:β_2-AR exhibited increased rates of protein synthesis as measured by acute puromycin incorporation[25] (Fig. 1d,e). To assess whether AAV:β_2-AR administration altered the muscle fibre type distribution, sections of treated TA muscles were examined *via* histochemical reaction to estimate succinate dehydrogenase (SDH) activity and immunolabelled for prevalence of the myosin type IIa isoform. Muscles examined four weeks after administration of AAV:β_2-AR or control vector did not exhibit a difference in the proportion of fibres expressing the type IIa myosin heavy chain isoform, or the activity of SDH (Supplementary Fig. S1).

To determine if the magnitude of hypertrophy induced *via* administration of AAV:β_2-AR was comparable to that achieved by treating muscles with anabolic β_2-AR agonists, additional cohorts of mice were administered AAV:β_2-AR, or daily injections of formoterol (100 μg/kg) for 28 days. We observed that a single administration of AAV:β_2-AR and 28 consecutive days of formoterol administration produced comparable increases in muscle mass (Fig. 1f, normalised relative to tibial bone length rather than body mass to account for the effect of changes in lean mass in mice receiving formoterol).

Muscle hypertrophy induced by β_2-AR gene delivery is not inhibited by rapamycin. As repeated administration of anabolic β_2-AR agonists has been reported to promote skeletal muscle growth *via* signalling dependent on the activation of mTOR, we investigated whether hypertrophy as a consequence of AAV:β_2-AR administration was also associated with mTOR-driven processes. Western blot analysis of TA muscles examined 14 days after administration of AAV:β_2-AR or control vector revealed a significant increase in phosphorylation of S6RP but not the upstream regulators Akt and mTOR (Supplementary Fig. S2). Additional mice administered AAV:β_2-AR were treated with 28 daily injections of rapamycin, an inhibitor of mTOR, to further test whether mTOR activity is necessary to achieve muscle hypertrophy associated with increased β_2-AR expression. Increases in muscle mass and myofibre diameter as a consequence of transducing muscles with AAV:β_2-AR did not differ between animals receiving rapamycin or vehicle for 28 days (Fig. 2a and Supplementary Fig. S3). Furthermore, whereas administration of AAV:β_2-AR increased phosphorylation of P70S6K and S6RP, rapamycin administration inhibited phosphorylation of these two proteins and 4EBP1 (Fig. 2b and Supplementary Fig. S3), thereby confirming the bioactivity of the rapamycin regimen used.

GαsL and Gαi2 gene delivery have opposing effects on TA muscle mass. As β_2-adrenoceptors utilise stimulatory (Gαs) and inhibitory (Gαi) G-proteins to propagate intracellular signalling, we investigated whether treating the TA muscles of mice with AAV vectors that increase expression of either GαsL or Gαi2 affected muscle mass. Injection of TA muscles with AAV:GαsL four weeks prior to examination increased mass by 8% (Fig. 3a), whereas administration of AAV:Gαi2 was associated with a 6% decrease in muscle mass (Fig. 3b). Expression of FLAG-tagged GαsL and Gαi2 proteins was confirmed by Western blot (Fig. 3c,d respectively). As our data showed Gαi2 to be a negative regulator of muscle mass, we investigated whether increased expression of Gαi2 could attenuate the anabolic effects of β_2-AR gene delivery. Cohorts of mice received intramuscular injections of AAV:β_2-AR in combination with AAV:Gαi2 or control vector. Consistent with effects reported in Fig. 1, mice administered AAV:β_2-AR and control vector demonstrated TA muscle hypertrophy (Fig. 4a). However, co-administration of AAV:Gαi2 completely prevented the anabolic effects of AAV:β_2-AR administration (Fig. 4a). To validate this observation, additional mice received bilateral TA muscle injections of AAV:β_2-AR in combination with AAV:Gαi2 or control vector. Four weeks after vector administration, TA muscles administered AAV:β_2-AR with control vector exhibited a 20% increased mass compared with contralateral muscles co-administered AAV:β_2-AR with AAV:Gαi2 (Fig. 4b). Immunolabelling of muscles confirmed comparable expression of β_2-AR between treatment conditions (Fig. 4c).

GαsXL gene delivery promotes muscle hypertrophy independent of β_1- and β_2- adrenoceptors. The extra-large isoform of Gαs, GαsXL, is predominantly expressed in neurons and has been reported to

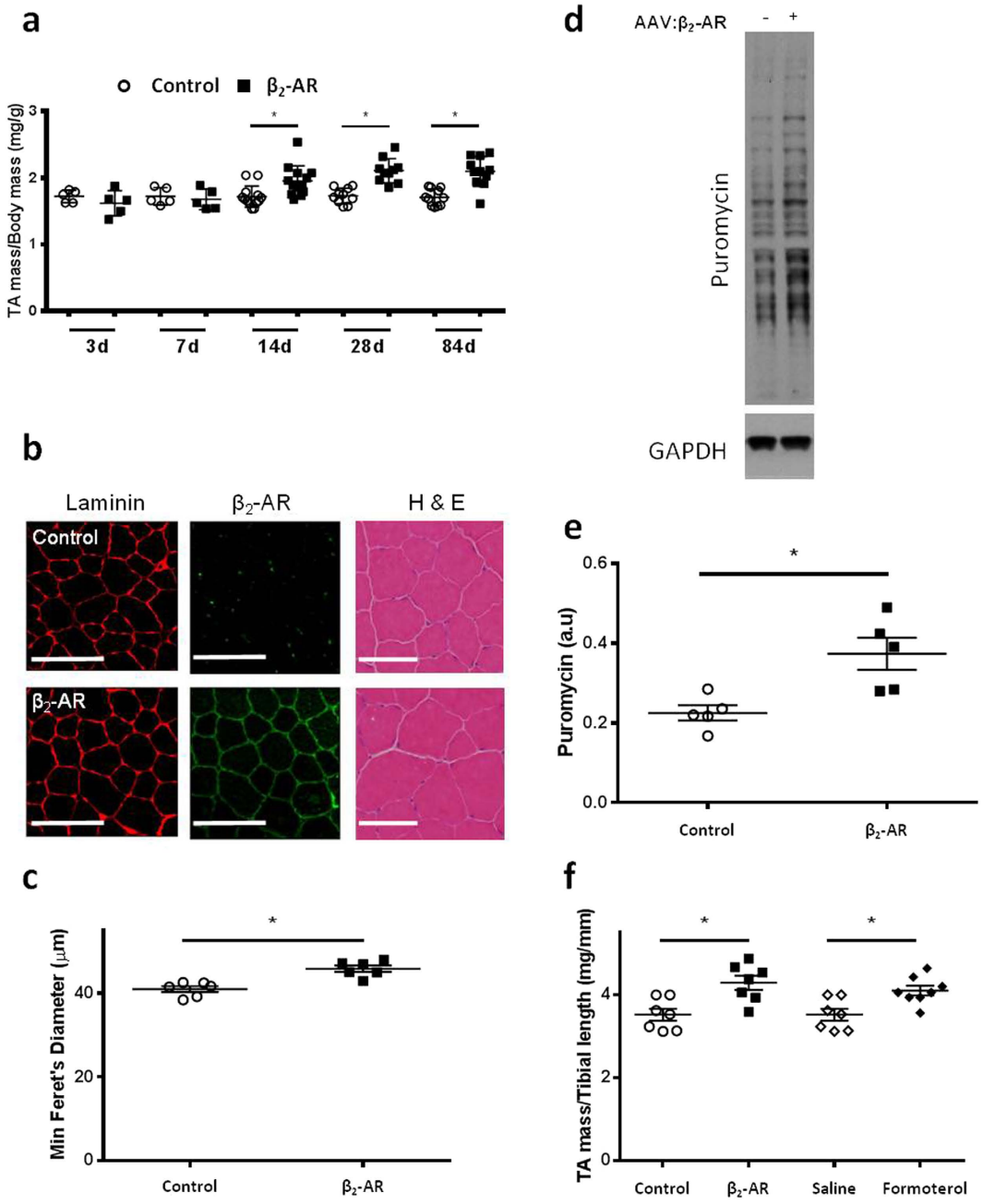

Figure 1. β_2-AR gene delivery promotes skeletal muscle hypertrophy and protein synthesis. (a) TA muscle mass examined 3, 7, 14, 28 and 84 days after AAV:β_2-AR administration. **(b)** Representative immunofluorescent images of β_2-AR density (shown in green) at the myofibre membrane (shown in red) in control and AAV:β_2-AR treated muscles four weeks post-injection (scale bar = 50 μm). Representative H&E images of TA muscle cross-sections four weeks after administration of control vector or AAV:β_2-AR (scale bar = 50 μm). **(c)** Minimum Feret's diameter measurements of TA muscle fibres examined four weeks after administration of control vector or AAV:β_2-AR. **(d,e)** Representative western blots and densitometry showing puromycin incorporation in TA muscles four weeks after vector administration. **(f)** TA muscle mass four weeks after administration of control vector or AAV:β_2-AR, or four weeks after 28 consecutive days of vehicle or formoterol administration (muscle mass is expressed relative to tibial bone length to account for differences in body mass caused by systemic effects of formoterol administration). Data are mean ± SEM. n = 3–10 mice/group. *$p < 0.05$.

promote increased cAMP activity compared to GαsL in a cell culture model of β_2-AR activation[26,27]. Reasoning that ectopic expression of GαsXL in skeletal muscle could confer greater effects on muscle mass than those achieved via GαsL gene delivery, we examined the effects of administering AAV:GαsXL to the TA muscles of C57Bl6 mice. Muscles examined 28 days after administration of AAV:GαsXL exhibited a 27% increase in mass

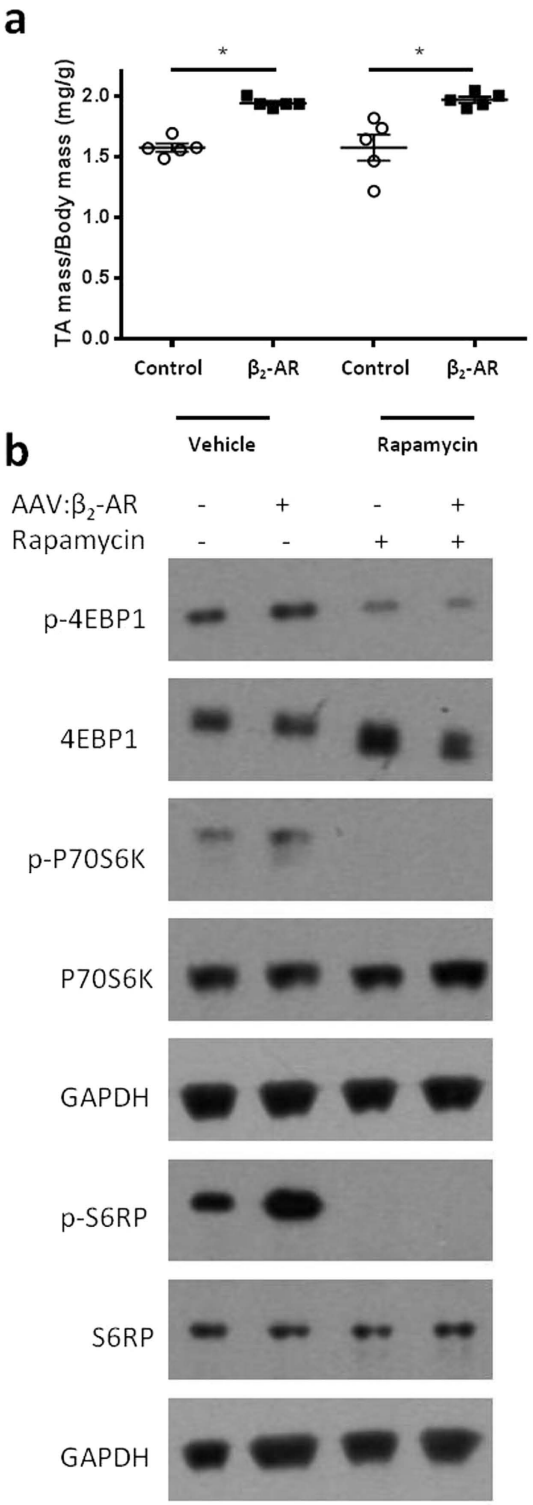

Figure 2. Muscle hypertrophy induced by β_2-AR gene delivery is not inhibited by Rapamycin. (a) TA muscle mass four weeks post control vector or AAV:β_2-AR injection and daily administration of vehicle or rapamycin. **(b)** Representative western blots indicating phosphorylated and total levels of 4EBP1, P70S6K, S6RP and GAPDH as a loading control. (n = 5 mice/group) Data are mean ± SEM. *$p < 0.05$.

(Fig. 5a) and a significant increase in myofibre diameter (Fig. 5b) compared to contralateral muscles receiving control vector. Administration of AAV:GαsXL was also associated with an increased proportion of muscle fibres expressing the type IIa myosin heavy chain isoform, although no accompanying significant change in SDH activity was observed (Supplementary Fig. S4). Consistent with the stimulatory effects of AAV:β_2-AR administration

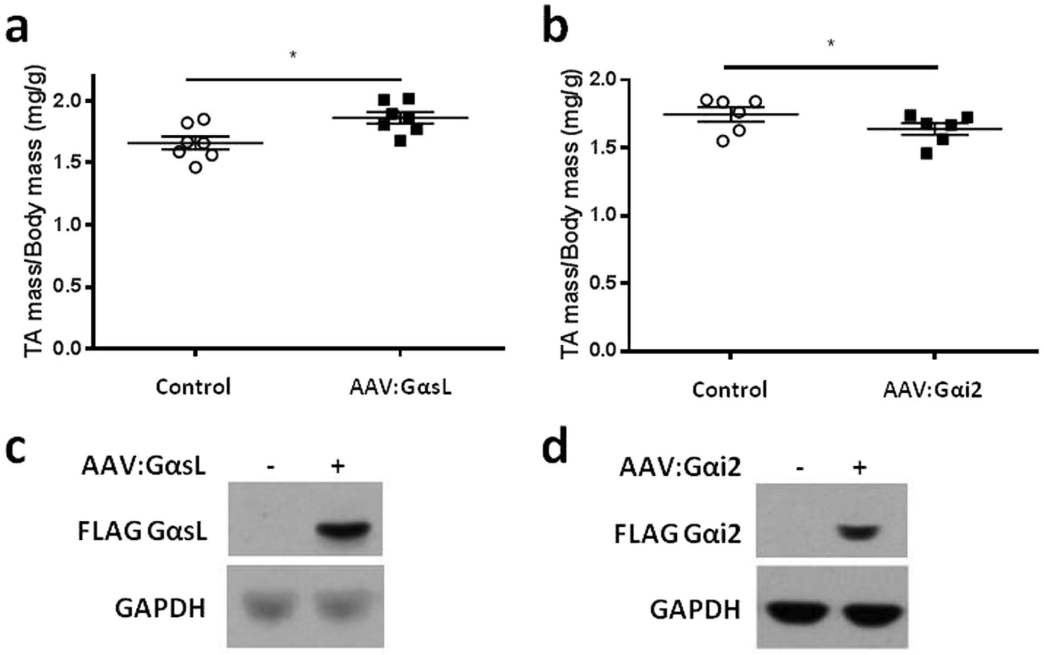

Figure 3. GαsL and Gαi2 gene delivery have opposing effects on TA muscle mass. (a) TA muscle mass four weeks after administration of control vector or AAV:GαsL. **(b)** TA muscle mass four weeks after administration of control vector or AAV:Gαi2. **(c)** Western blot analysis of FLAG-tagged GαsL in TA muscles injected with AAV:GαsL. **(d)** Western blot analysis of FLAG-tagged Gαi2 in TA muscles injected with AAV:Gαi2. Data are mean ± SEM. n = 6–7 mice/group. *$p < 0.05$.

upon protein synthesis (reported in Fig. 1d), the muscles of wild-type mice treated with AAV:GαsXL also demonstrated markedly increased rates of protein synthesis, as estimated from puromycin incorporation (Fig. 5e,f). To determine whether muscle hypertrophy associated with AAV:GαsXL administration was dependent on β-AR activity, we administered AAV:GαsXL to mice lacking functional β_1- and β_2-ARs (β_1/β_2^{mut} mice)[28,29]. Four weeks after administration of AAV:GαsXL to β_1/β_2^{mut} mice, treated TA muscles exhibited a 35% increased mass (Fig. 5a), and significantly increased muscle fibre diameter (Fig. 5b,c), compared with contralateral muscles administered control vector. Comparable expression of GαsXL was confirmed in the treated muscles of C57Bl6 and β_1/β_2^{mut} mice by western blot probing for flag-tagged GαsXL (Fig. 5d).

Discussion

Although synthetic β_2-AR agonists exert anabolic and anti-catabolic effects on mammalian skeletal muscles, their clinical application for muscle wasting has been limited by concerns regarding potential off-target effects[30–32]. Our findings demonstrate a novel method of stimulating β_2-AR signalling in muscle fibres, based on the use of recombinant AAV vectors to deliver β_2-AR or Gαs expression constructs. As recombinant viral vectors can be configured to achieve tissue-specific transgene delivery and expression by combining the cell-selective tropism of vectors with cell-specific transcription/translation control elements[33,34], muscle-directed gene delivery may hold potential as a strategy for manipulating the β_2-adrenergic network without the need to repeatedly administer potent β_2-AR agonists. The benefits of such an approach could provide the means to effectively promote anabolic signalling in the target tissue (i.e. skeletal muscle), while minimising the potential for incurring off-target effects in other tissues.

Using rAAV vectors to enhance β_2-AR expression in mouse limb muscles promoted increases in myofibre size and augmented protein synthesis. The hypertrophic effects of AAV:β_2-AR administration were comparable in magnitude to those achieved with repeated administration of the potent β_2-AR agonist formoterol. We did not find evidence that activation of mTOR was required to support muscle hypertrophy induced by β_2-AR gene delivery, which contrasts with reports of β_2-AR agonist-induced skeletal muscle hypertrophy requiring mTOR signalling[9]. As myogenic cells can elicit an anabolic response downstream of the β_2-AR *via* the PKC/GSK3β signalling axis[35], hypertrophy as a result of AAV:β_2-AR administration could utilise similar mechanisms. These observations point to other possible advantages of developing skeletal-muscle-directed gene delivery as an alternative method for manipulating β_2-adrenergic signalling *in vivo*. Further research is warranted to more comprehensively examine the similarities and differences between drug- and gene-based interventions targeting this signalling system in striated muscle.

Having established that β_2-AR gene delivery can stimulate skeletal muscle hypertrophy, we investigated whether β_2-AR-mediated effects could be potentiated by increasing the abundance of specific Gα protein subunits operating as signalling substrates for the β_2-AR. Increasing expression of GαsL promoted a modest hypertrophic effect compared with AAV:β_2-AR administration. Broadly, this stimulatory role of Gαs is consistent with

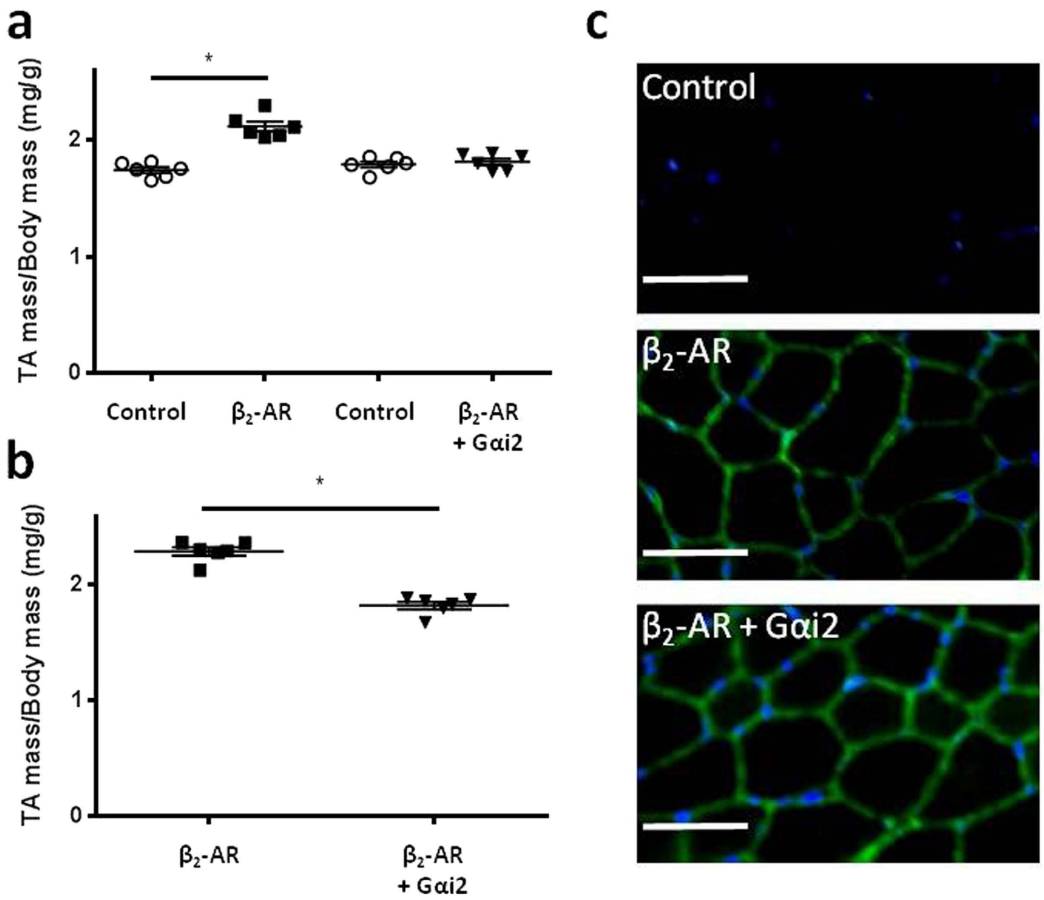

Figure 4. Gαi2 gene delivery inhibits the anabolic effects of β_2-AR gene delivery. **(a)** TA muscle mass four weeks after administration of control vector, AAV:β_2-AR or AAV:β_2-AR and AAV:Gαi2. **(b)** TA muscle mass four weeks after administration of AAV:β_2-AR with control vector or AAV:β_2-AR with AAV:Gαi2. **(c)** Representative immunofluorescent images confirming β_2-AR over-expression (shown in green) and nuclei (shown in blue) four weeks after vector administration (scale bar = 50 μm). Data are mean ± SEM. n = 6 mice/group. *$p < 0.05$.

earlier work demonstrating that muscle mass is reduced in Gαs knockout mice[36]. In contrast, increasing expression of Gαi2 alone produced muscle atrophy, and more significantly, co-delivery of AAV:Gαi2 with AAV:β_2-AR completely prevented the hypertrophic effects of β_2-AR gene delivery. These findings are consistent with the possibility that Gαi2 possesses comparatively greater (relative to Gαs) affinity for interaction with the β_2-AR, or that increased abundance of Gαi2 can outcompete Gαs for interaction with the β_2-AR.

Although Gαi2 is considered to operate in opposition to Gαs, the inhibitory effects of over-expressing wild-type Gαi2 in muscle fibres described herein contrast with studies that documented protein accretion and cell growth after transducing myogenic cells with a constitutively active $G\alpha i2^{Q205L}$ mutant[35]. Global embryonic knock-out of Gαi2 produces mice with muscles of reduced myofibre size, although the animals also suffer from a lethal intestinal phenotype and immune cell defects that likely compromise interpretation of the muscle attributes[37,38]. Stronger evidence from cell culture studies supports a role for Gαi2 in guiding the proliferation and differentiation of myogenic progenitors[35,37]. While the mechanisms by which $G\alpha i2^{Q205L}$ promotes cell proliferation and recruitment lie outside the scope of the present study, the differences in effects of $G\alpha i2^{Q205L}$ reported elsewhere versus the effects of wild-type Gαi2 reported here appear to be attributed at least in part to differential actions within myogenic progenitor cells versus muscle fibres. Additionally, it cannot yet be ruled out that the $G\alpha i2^{Q205L}$ mutant isoform does not exert different effects on downstream signalling targets or is affected differently by feedback mechanisms.

Although the GNAS gene encodes for the GαsL G-protein in skeletal muscle, alternate Gαs transcript variants are encoded by GNAS in other cell types. As the predominantly neuroendocrine GαsXL variant[39] has been reported to stimulate increased cAMP activity when compared with GαsL[26,27], we reasoned that expressing GαsXL in muscle fibres may cause a hypertrophic response in skeletal muscle. Supporting this hypothesis, we found that muscles treated with AAV:GαsXL demonstrated a significant hypertrophic response with effects comparable to those achieved by treating muscles with either AAV:β_2-AR or formoterol. Muscles treated with AAV:GαsXL exhibited an increased proportion of myofibres expressing the type IIa myosin heavy chain isoform, whereas no such effect was noted in muscles receiving AAV:β_2-AR. These findings lend support to the idea that

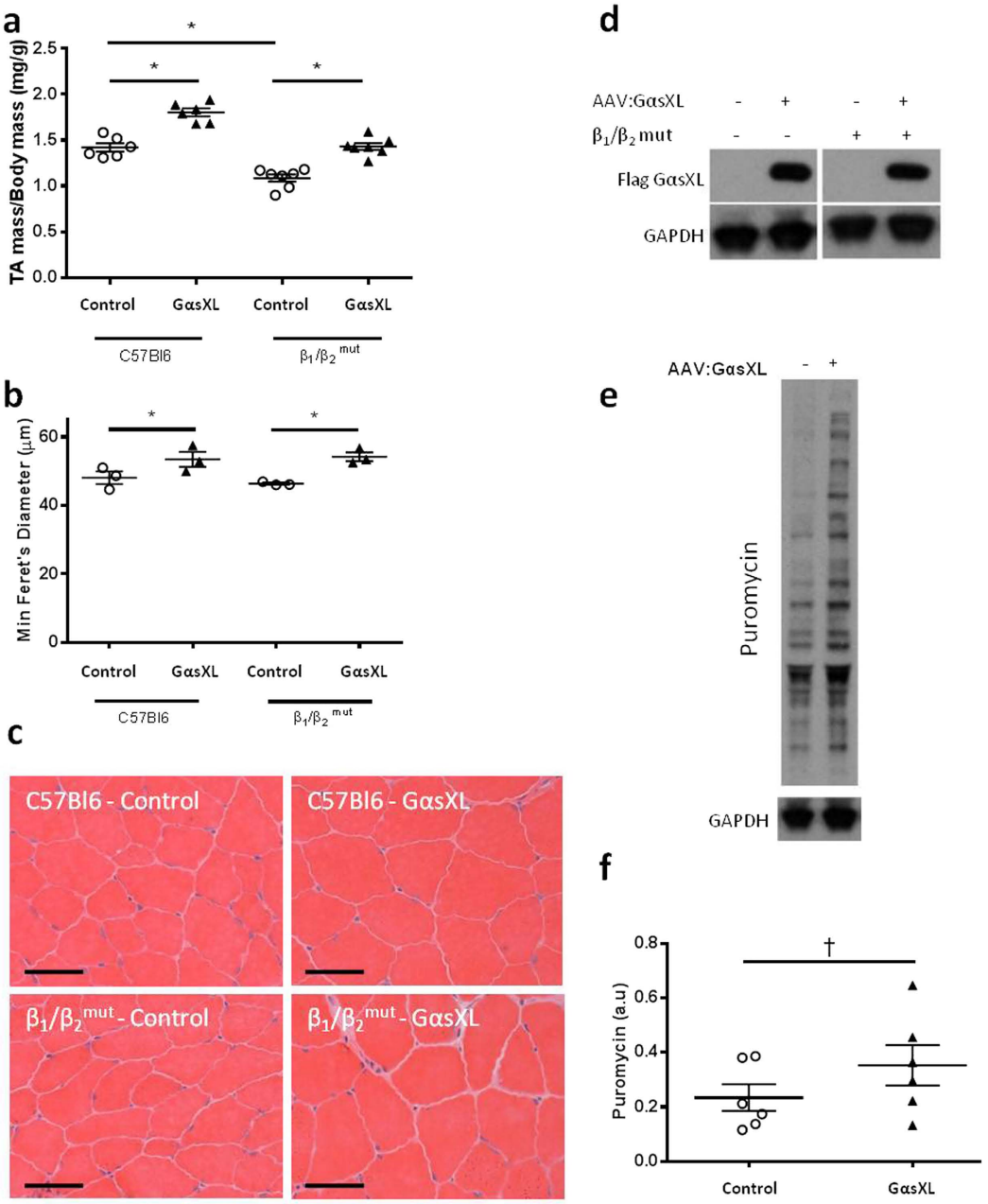

Figure 5. GαsXL gene delivery promotes muscle hypertrophy independent of β_1- and β_2- adrenoceptors. **(a)** TA muscle mass four weeks after administration of control vector or AAV:GαsXL to C57Bl6 or β_1/β_2^{mut} mice. **(b)** Minimum Feret's diameter measurements of TA muscle fibres from C57Bl6 and β_1/β_2^{mut} mice treated with control vector or AAV:GαsXL. **(c)** Representative H&E images of TA muscle cross-sections examined four weeks after vector administration (scale bar = 50 μm). **(d)** Western blot confirming the presence of flag tagged GαsXL in treated muscles. **(e,f)** Western blot and densitometry displaying puromycin incorporation in TA muscles four weeks post treatment. Data points are portrayed together with mean ± SEM. n = 3–6 mice/group. $^{*}p < 0.05$. $^{\dagger}p = 0.05$.

the two vector-based interventions have differing effects upon the physiological properties of treated skeletal muscles. The marked muscle hypertrophy with administration of AAV:GαsXL was recapitulated in mice lacking functional β_1- and β_2-ARs, which do not exhibit an anabolic response when administered anabolic β-agonists[40]. These results demonstrate that expression of GαsXL in skeletal musculature confers anabolic adaptations that are not dependent on active β_1- and β_2-ARs. It is not clear whether the observed muscle hypertrophy is a product of GαsXL possessing constitutive activity, or whether GαsXL proteins may be activated by other GPCRs in muscle fibres. Other receptors in muscle that could function as activators of ectopically expressed GαsXL include Fzd7 (previously implicated in regulation of myogenic cells[41]) and PTH1 (which promotes GαsXL activation in other

tissues[42,43]). Collectively, these findings highlight fascinating aspects of how G proteins can modulate muscle attributes, and support the rationale for further study.

In summary, this study presents the first demonstration that treatment of mammalian skeletal muscle fibres with recombinant AAV vectors expressing the β_2-AR or GαsXL promote changes in protein turnover that favour myofibre hypertrophy. These proof-of-concept studies focused on manipulating β-adrenergic signalling in individual limb muscles, and demonstrate the feasibility of stimulating anabolic signalling *via* the β_2-AR signalling pathway without administering β_2-AR agonists. The findings provide important insight into GPCR signalling in skeletal muscle, with implications for developing novel interventions for muscle wasting conditions. Given the uncertainties regarding the long-term administration of potent β_2-AR agonists to patients, developing new strategies by which to promote anabolic β_2-AR signalling in skeletal muscle without using β_2-AR agonists warrants deeper investigation. This includes systemic administration of AAV vectors to achieve body-wide transduction of skeletal muscles. The findings reported here provide valuable insight into a new intervention concept, upon which such studies could be developed. Comprehensively investigating the consequences of muscle-directed gene delivery in mouse models of muscle wasting will help to determine the therapeutic potential of this novel strategy, including effects on muscle functionality and other organ systems.

Methods and Materials

All reagents were purchased from Sigma-Aldrich unless otherwise stated.

Animal Experiments. *In vivo* procedures were conducted in accordance with the relevant codes of practice for the care and use of animals for scientific purposes (National Institute of Health, 1985, and the National Health & Medical Research Council of Australia 2013). All experimental protocols were approved by the Alfred Medical and Education Precinct Animal Ethics Committee (AMREP AEC). All surgical procedures were performed under inhalation of isoflurane in medical oxygen with post-operative analgesia. Eight to 10 week old, male, C57Bl/6 and β_1/β_2 mutant (β_1/β_2^{mut}) mice were used for all experiments. Animals were fed standard chow diets with access to drinking water *ad libitum* while housed under a 12-hour light dark cycle. β_1/β_2^{mut} mice were sourced and bred as described previously[28]. Doses of AAV:β_2-AR, AAV:Gαi2, AAV:GαsL (1×10^{10} vg) and AAV:GαsXL (1×10^9 vg) vectors (identified from preliminary dose-optimisation experiments) were diluted in 30 µl of Hank's buffered saline solution (HBSS) and directly injected into the TA muscle. Control injections consisted of the administration of a viral vector lacking a functional gene into the contralateral limb. For systemic β-agonist treatments, intraperitoneal injections of formoterol at 100 µg/kg or saline were administered daily for 28 days. Rapamycin (ApexBio) was dissolved in DMSO to a stock concentration of 10 mg/ml, and a working concentration of rapamycin was formulated in a solution of 0.1% carboxymethylcellulose and 0.125% polysorbate-80. Mice received 2 mg/kg of rapamycin one day before and at the time of AAV:β_2-AR administration by intraperitoneal injection. Mice were treated daily until experimental endpoint. For puromycin administration, mice received 0.04 µmol/g of puromycin (Life Technologies) via intraperitoneal injection exactly 30 min before experimental endpoint. Experimental endpoints were 28 days post viral vector administration unless indicated otherwise. Mice were humanely killed *via* cervical dislocation and the muscles rapidly excised and weighed before subsequent processing.

Antibodies. All antibodies were purchased from Cell Signaling Technologies and used at a dilution of 1:1000, except anti-puromycin and anti-laminin B2 (Millipore) which were used at 1:5000 and 1:250 respectively, anti-β_2-AR (MBL) 1:500 and anti-GAPDH (Santa Cruz Biotechnology) 1:10000.

Recombinant AAV vector design and production. Traditional cloning techniques were used to generate cDNA constructs encoding Adrb2 (β_2-AR), Gnai2 (Gαi2), GnasL (GαsL) and GnasXL (GαsXL) (synthesized by GenScript) which were cloned into an AAV expression plasmid consisting of a cytomegalovirus (CMV) promoter and SV40 poly-A region flanked by AAV2 terminal repeats. The Gαi2, GαsL and GαsXL cDNA construct also included a flag-tag coding region at the 5′ end of the coding sequences. Viral vector production was performed as described previously[21]. Briefly, HEK-293 cells were plated at a density of $3.2–3.8 \times 10^6$ cells on a 10 cm culture dish, 8–16 hours before transfection with 10 µg of a vector genome-containing plasmid and 20 µg of the packaging/helper plasmid pDGM6 by calcium phosphate precipitation. At 72 hours post transfection, the medium and cells were collected and homogenized through a microfluidizer (Microfluidics) before 0.22 µm clarification (Millipore). Purification of viral particles from crude lysates was performed using affinity chromatography over a heparin affinity column (HiTrap, Amersham), and ultracentrifugation overnight prior to re-suspension in sterile physiological Ringer's solution. The purified vector preparations were titered with a customized sequence-specific quantitative PCR-based reaction (Life Technologies).

Western blotting. Muscles were homogenized in NP-40 lysis buffer containing protease and phosphatase inhibitor cocktails. Lysates were centrifuged at 15,000 g for 20 min at 4 °C, protein concentration was determined using a BCA protein assay kit (Thermo Scientific) and samples denatured for 5 min at 95 °C. Protein fractions were resolved by SDS-PAGE using pre-cast 4–12% Bis-Tris gels (Life Technologies), blotted onto nitrocellulose membranes (BioRad) and incubated with the appropriate primary antibody and detected as described previously[44]. Quantification of labelled western blots was performed using ImageJ pixel analysis (NIH Image software), and data normalized to corresponding GAPDH controls.

Histological analysis. Harvested muscles were embedded in optimum cutting temperature (OCT) cryoprotectant (Sakura Finetek) and frozen in liquid nitrogen-cooled isopentane. Frozen samples were cryosectioned at 10 µm thickness using a Leica CM1950 cryostat. Cross-sections were fixed in room temperature methanol and stained with hematoxylin and eosin as described previously[44]. Stained sections of muscles were examined using

a light microscope with digital camera (BX-50, Olympus), to capture images analysed for muscle fibre morphology. Minimum Feret's diameter of myofibres was quantified using ImageJ software analysis. Up to eight fields of view were captured from the same locations within each TA muscle and contrast adjusted to gate fibres based on numerical threshold, >200 myofibres were measured per muscle. Histochemical estimation of SDH activity and immunolabelling of the type-IIa myosin heavy chain isoform was performed on 10 µm thick cryosections of harvested TA muscles as previously described[45]. Images were captured (Axio Imager D1 microscope, Carl Zeiss). SDH activity was estimated by capturing four 100x magnification brightfield images per TA muscle, and quantifying pixel density for each muscle fibre within the identified fields *via* ImageJ software analysis. Myosin type IIa positive fibres were counted and expressed relative to total number of myofibres counted per section (>600 fibres samples per muscle).

Immunofluorescence Microscopy. OCT-frozen TA samples were cryosectioned at 8 µm thickness, fixed in methanol, washed in potassium phosphate buffered saline (KPBS) containing gelatin and blocked in a solution consisting of Tween-20, BSA, gelatin and KPBS. The sections were incubated in anti-laminin B2 and anti-β_2-AR primary antibodies overnight at 4 °C. Alexa-Fluor-488 and -594 secondary goat antibodies (Life Technologies) were used to detect β_2-AR and laminin B2 primary antibodies respectively, followed by 3 min incubation in DAPI nuclear stain (Life Technologies) and mounting in HardSet Vectashield (Vector Laboratories). Images were captured using a BX61 light microscope (Olympus).

Statistical Analysis. All data are represented as the mean ± SEM. A paired Student's t-test was used for comparisons between two conditions. Two-way analysis of variance (ANOVA) was used to measure statistical differences between multiple conditions with Tukey's *post hoc* analysis for specific group comparisons. All significant differences are reported with $p < 0.05$.

References

1. Premont, R. T. & Gainetdinov, R. R. Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu. Rev. Physiol.* **69,** 511–534 (2007).
2. Dror, R. O. *et al.* Activation mechanism of the β_2-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **108,** 18684–18689 (2011).
3. Ryall, J. G., Church, J. E. & Lynch, G. S. Novel role for β-adrenergic signalling in skeletal muscle growth, development and regeneration. *Clin. Exp. Pharmacol. Physiol.* **37,** 397–401 (2010).
4. Daaka, Y., Luttrell, L. M. & Lefkowitz, R. J. Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390,** 88–91 (1997).
5. Pearen, M. A., Ryall, J. G., Lynch, G. S. & Muscat, G. E. Expression profiling of skeletal muscle following acute and chronic β_2-adrenergic stimulation: implications for hypertrophy, metabolism and circadian rhythm. *BMC Genomics* **10,** 448 (2009).
6. Ryall, J. G., Plant, D. R., Gregorevic, P., Sillence, M. N. & Lynch, G. S. β_2-agonist administration reverses muscle wasting and improves muscle function in aged rats. *J. Physiol. (Lond.)* **555,** 175–188 (2004).
7. Toledo, M. *et al.* Formoterol in the treatment of experimental cancer cachexia: effects on heart function. *J. Cachexia Sarcopenia Muscle* **5,** 315–320 (2014).
8. Rommel, C. *et al.* Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat. Cell Biol.* **3,** 1009–1013 (2001).
9. Kline, W. O., Panaro, F. J., Yang, H. & Bodine, S. C. Rapamycin inhibits the growth and muscle-sparing effects of clenbuterol. *J. Appl. Physiol.* **102,** 740–747 (2007).
10. Gonçalves, D. A. *et al.* Mechanisms involved in 3′, 5′-cyclic adenosine monophosphate-mediated inhibition of the ubiquitin-proteasome system in skeletal muscle. *Endocrinology* **150,** 5395–5404 (2009).
11. Joassard, O. R. *et al.* Regulation of Akt-mTOR, ubiquitin-proteasome and autophagy-lysosome pathways in response to formoterol administration in rat skeletal muscle. *Int. J. Biochem. Cell Biol.* **45,** 2444–2455 (2013).
12. Koopman, R. *et al.* Cellular mechanisms underlying temporal changes in skeletal muscle protein synthesis and breakdown during chronic β-adrenoceptor stimulation in mice. *J. Physiol. (Lond.)* **588,** 4811–4823 (2010).
13. Maltin, C. A. *et al.* Inhibition and reversal of denervation-induced atrophy by the β-agonist growth promoter, clenbuterol. *Biosci. Rep.* **6,** 811–818 (1986).
14. Jiang, G. L. *et al.* Randomized, double-blind, and placebo-controlled trial of clenbuterol in denervated muscle atrophy. *ISRN Pharmaceut.* **2011,** 981254 (2011).
15. Harcourt, L. J., Schertzer, J. D., Ryall, J. G. & Lynch, G. S. Low dose formoterol administration improves muscle function in dystrophic *mdx* mice without increasing fatigue. *Neuromus. Dis.* **17,** 47–55 (2007).
16. Kissel, J. T. *et al.* Pilot trial of albuterol in facioscapulohumeral muscular dystrophy. FSH-DY Group. *Neurology* **50,** 1402–1406 (1998).
17. Skura, C. L., Fowler, E. G., Wetzel, G. T., Graves, M. & Spencer, M. J. Albuterol increases lean body mass in ambulatory boys with Duchenne or Becker muscular dystrophy. *Neurology* **70,** 137–143 (2008).
18. Ryall, J. G., Schertzer, J. D. & Lynch, G. S. Attenuation of age-related muscle wasting and weakness in rats after formoterol treatment: therapeutic implications for sarcopenia. *J. Gerontol. A. Biol. Sci. Med. Sci.* **62,** 813–823 (2007).
19. Costelli, P. *et al.* Muscle protein waste in tumor-bearing rats is effectively antagonized by a β_2-adrenergic agonist (clenbuterol). Role of the ATP-ubiquitin-dependent proteolytic pathway. *J. Clin. Invest.* **95,** 2367–2372 (1995).
20. Greig, C. A. *et al.* Phase I/II trial of formoterol fumarate combined with megestrol acetate in cachectic patients with advanced malignancy. *Support. Care Cancer* **22,** 1269–1275 (2014).
21. Blankinship, M. J. *et al.* Efficient transduction of skeletal muscle using vectors based on adeno-associated virus serotype 6. *Mol. Ther.* **10,** 671–678 (2004).
22. Gregorevic, P. *et al.* Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nat. Med.* **10,** 828–834 (2004).
23. Mendell, J. R. *et al.* A phase 1/2a follistatin gene therapy trial for becker muscular dystrophy. *Mol. Ther.* **23,** 192–201 (2015).
24. Buchlis, G. *et al.* Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. *Blood* **119,** 3038–3041 (2012).
25. Goodman, C. A. *et al.* Novel insights into the regulation of skeletal muscle protein synthesis as revealed by a new nonradioactive *in vivo* technique. *FASEB J.* **25,** 1028–1039 (2011).
26. Linglart, A. *et al.* Coding GNAS mutations leading to hormone resistance impair *in vitro* agonist- and cholera toxin-induced adenosine cyclic 3′,5′-monophosphate formation mediated by human XLαs. *Endocrinology* **147,** 2253–2262 (2006).
27. Mariot, V. *et al.* Potent constitutive cyclic AMP-generating activity of XLαs implicates this imprinted GNAS product in the pathogenesis of McCune-Albright syndrome and fibrous dysplasia of bone. *Bone* **48,** 312–320 (2011).

28. Church, J. E. *et al.* Functional β-adrenoceptors are important for early muscle regeneration in mice through effects on myoblast proliferation and differentiation. *PLoS One* **9,** e101379 (2014).
29. Rohrer, D. K., Chruscinski, A., Schauble, E. H., Bernstein, D. & Kobilka, B. K. Cardiovascular and metabolic alterations in mice lacking both beta1- and beta2-adrenergic receptors. *J. Biol. Chem.* **274,** 16701–16708 (1999).
30. Burniston, J. G. *et al.* Myotoxic effects of clenbuterol in the rat heart and soleus muscle. *J. Appl. Physiol.* **93,** 1824–1832 (2002).
31. Duncan, N. D., Williams, D. A. & Lynch, G. S. Deleterious effects of chronic clenbuterol treatment on endurance and sprint exercise performance in rats. *Clin. Sci.* **98,** 339–347 (2000).
32. Gregorevic, P., Ryall, J. G., Plant, D. R., Sillence, M. N. & Lynch, G. S. Chronic β-agonist administration affects cardiac function of adult but not old rats, independent of β-adrenoceptor density. *Am. J. Physiol. Heart Circ. Physiol.* **289,** H344–349 (2005).
33. Brown, B. D. *et al.* Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat. Biotechnol.* **25,** 1457–1467 (2007).
34. Salva, M. Z. *et al.* Design of tissue-specific regulatory cassettes for high-level rAAV-mediated expression in skeletal and cardiac muscle. *Mol. Ther.* **15,** 320–329 (2007).
35. Minetti, G. C. *et al.* Gαi2 signaling promotes skeletal muscle hypertrophy, myoblast differentiation, and muscle regeneration. *Sci. Signaling* **4,** ra80 (2011).
36. Chen, M. *et al.* $G_s\alpha$ deficiency in skeletal muscle leads to reduced muscle mass, fiber-type switching, and glucose intolerance without insulin resistance or deficiency. *Am. J. Physiol. Cell Physiol.* **296,** C930–940 (2009).
37. Minetti, G. C. *et al.* Gαi2 signaling is required for skeletal muscle growth, regeneration, and satellite cell proliferation and differentiation. *Mol. Cell. Biol.* **34,** 619–630 (2014).
38. Rudolph, U. *et al.* Ulcerative colitis and adenocarcinoma of the colon in Gαi2-deficient mice. *Nat. Genet.* **10,** 143–150. (1995).
39. Weinstein, L. S., Xie, T., Zhang, Q. H. & Chen, M. Studies of the regulation and function of the $G_s\alpha$ gene Gnas using gene targeting technology. *Pharmacol. Ther.* **115,** 271–291 (2007).
40. Hinkle, R. T. *et al.* Skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol are mediated by the β_2-adrenergic receptor. *Muscle Nerve* **25,** 729–734 (2002).
41. von Maltzahn, J., Bentzinger, C. F. & Rudnicki, M. A. Wnt7a-Fzd7 signalling directly activates the Akt/mTOR anabolic growth pathway in skeletal muscle. *Nat. Cell Biol.* **14,** 186–191 (2012).
42. He, Q., Zhu, Y., Corbin, B. A., Plagge, A. & Bastepe, M. The G protein α subunit variant $XL\alpha_s$ promotes inositol 1,4,5-trisphosphate signaling and mediates the renal actions of parathyroid hormone *in vivo*. *Sci Signal* **8,** ra84 (2015).
43. Kimura, S. & Yoshioka, K. Parathyroid hormone and parathyroid hormone type-1 receptor accelerate myocyte differentiation. *Sci. Rep.* **4,** 5066 (2014).
44. Winbanks, C. E. *et al.* TGF-β regulates miR-206 and miR-29 to control myogenic differentiation through regulation of HDAC4. *J. Biol. Chem.* **286,** 13805–13814 (2011).
45. Johnston, A. J. *et al.* Targeting of Fn14 Prevents Cancer-Induced Cachexia and Prolongs Survival. *Cell* **162,** 1365–1378 (2015).

Acknowledgements

The authors thank Dr Catherine E. Winbanks and Dr Lucy Cassar (previously Baker IDI Heart and Diabetes Institute), Mr Timur Naim (Department of Physiology, University of Melbourne) and Mr Stephen Cody and Dr Iska Carmichael (Micro Imaging facility, AMREP campus) for technical guidance. This work was supported by Project Grant funding (509313; 1026231 awarded to G.S.L. and P.G.) from the National Health and Medical Research Council (NHMRC). P.G. is supported by a NHMRC Career Development Fellowship (1046782) and previously, a Senior Research Fellowship sponsored by Pfizer Australia. The Baker IDI Heart & Diabetes Institute is supported in part by the Operational Infrastructure Support Program of the Victorian Government. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

Designed the studies: A.H., G.S.L., P.G. Undertook the experimental work: A.H., T.D.C., R.E.T., H.Q., P.G. Analysed the data A.H., T.D.C., R.E.T., G.S.L., P.G. Contributed to figure and manuscript preparation: A.H., T.D.C., G.S.L., P.G.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Hagg, A. *et al.* Using AAV vectors expressing the β_2-adrenoceptor or associated Gα proteins to modulate skeletal muscle mass and muscle fibre size. *Sci. Rep.* **6**, 23042; doi: 10.1038/srep23042 (2016).