Activin-βC modulates cachexia by repressing the ubiquitin-proteasome and autophagic degradation pathways

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Abstract

Background Cancer-associated cachexia and muscle wasting are considered key determinants of cancer-related death and reduction in the quality of life of cancer patients. A crucial link has been established between activin signaling and skeletal muscle atrophy-hypertrophy.

We previously showed that activin-βC, a novel activin-A antagonist, is a tumor modulator that abolishes the cancer-associated cachexia in a mouse genetic model of gonadal tumorigenesis, in which the normal balance of inhibin/activin signalling is disrupted by a targeted mutation in the Inha gene (inhibin α-KO mouse). This study aimed to identify the molecular mechanism by which activin-βC increases survival and abolishes cancer-associated cachexia in α-KO mice. We hypothesized that overexpression of activin-βC modulates the cachexia phenotype by antagonizing the activin signaling pathway and repressing muscle wasting via the ubiquitin-proteasome and the autophagic-lysosomal degradation pathways.

Methods Male and female ActC++, α-KO, and α-KO/ActC++ mice and WT littermate controls were studied. Western blot analysis for the specific E3 ubiquitin ligases, atrogin-1 and MuRF1, markers of the autophagic-lysosomal pathway, Beclin-1, p62, and LC3A/B, effectors Smad-2, Smad-3 and myostatin was performed in the gastrocnemius of age-matched mice. Histopathology of the gastrocnemius and survival analysis were also conducted in animals from the same breeding cohort. Serum levels of activin-A, inflammatory cytokines, hormonal profile, and bone density were also assessed.

Results Increased levels of atrogin-1, MuRF-1, Beclin-1, p62, LC3A/B-I, Smad-2 and serum levels of activin-A were noted in the α-KO mice. These mice developed gonadal cancers followed by severe weight loss, and reduced survival. Overexpression of activin-βC antagonized the activin signaling cascade, attenuating the ubiquitin-proteasome and the autophagic-lysosomal degradation pathways, and reduced serum levels of activin-A. α-KO/ActC++ mice displayed a less aggressive cachectic phenotype, reduced tumor weight, and prolonged survival.

Conclusion Our findings show for the first time a specific effect of activin-βC on muscle wasting and transcription factors involved in muscle protein degradation. The study indicates that activin-βC may be a novel therapy to abrogate cancer-associated weight loss and prolong survival.

Introduction Cancer cachexia has been recently defined as a syndrome affecting the majority of cancer patients with advanced cancer and is associated with a reduction in treatment tolerance, response to therapy, quality of life, and survival. The clinical manifestation of cachexia is characterized by skeletal muscle wasting with or without loss of fat mass, and it is often associated with psychological distress, fatigue, and deterioration in physical function.5
Management of cancer cachexia is clinically challenging because of the absence of established therapeutic protocols to treat this multifaceted syndrome. Only two therapies, resulting from randomized trials, are available to treat cancer-associated cachexia: corticosteroids and progestins. However, neither of these drugs has a significant effect on muscle loss, and the side effects associated with their administration limit long-term use. Development of new drugs to target cancer cachexia is extremely difficult because of the complicated pathogenesis of this condition.

Several hormones, cytokines, and tumor-derived factors have been shown to influence the pathogenesis of muscle wasting and cancer-associated cachexia. For example, inflammation and inflammatory response to the tumor are factors participating in the development of cancer-associated cachexia. In the last two decades, different catabolic mediators (both humoral and tumoral) involved in cancer have been considered as targets for clinical investigations and/or therapeutic strategies without a significant improvement in the clinical management of cancer-associated weight loss.

The TGF-β family of ligands, including myostatin, activin-A, and Growth Differentiation Factor 11 (GDF11) and the receptors mediating signaling in particular the ActRIIB (a high-affinity activin type-II receptor in muscle), have been shown to have a crucial role in regulating muscle growth. Additionally, in the inhibin-α deficient mouse model (α-KO), where activins are deregulated because of the loss of the inhibin-α subunit, gonadal tumours and a cachexia phenotype can be observed. Lee and co-workers provided the first demonstration that the soluble receptor ActRIIB induces muscle hypertrophy in vivo. Additionally, Klimek and colleagues showed that preservation of muscle wasting could be obtained using a soluble form of the ActRIIB. Zhou et al. have showed the potential therapeutic benefit of blocking the activin signalling through ActRIIB in cancer cachexia. Administration of a decoy receptor to antagonize the ActRIIB pathway in four distinct models of lethal cancer cachexia vs. control mice, which were homozygous for a null allele of the ActRIIB gene, showed a significant reduction in the incidence of cachexia-like symptoms and increased survival rates. These findings suggested that the activin-βC subunit plays an important role in activin-A antagonism.

Therefore, this study was designed to determine the molecular mechanism by which activin-βC antagonized cancer cachexia and increased survival in the α-KO mice. Specifically, we aimed to determine whether overexpression of activin-βC was sufficient to regulate protein expression of factors involved in protein degradation, focusing our attention on the ubiquitin-proteasome catabolic system and the autophagic-lysosomal pathways. We also explored the impact of activin-βC on the inflammatory (IL-6, IFN-γ, and TNF-α) and hormonal profile of these mice, and we assessed its impact on bone density.

### Material and methods

**Experimental animals**

All experiments were approved by the Animal Ethics Committee of the University of Otago and conducted in accordance with the New Zealand code of practice in adherence with the National Institutes of Health (NIH) guide for the care and use of laboratory animals. All animals were housed under a 12:12 h light–dark cycle, food and water were available ad libitum. Mice on C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the University of Otago. α-KO mice were kindly provided by Professor Martin Matzuk (Baylor College of Medicine Houston).

Human activin-βC (under the control of a CMV promoter) - overexpressing mice (ActC++) were obtained from Monash University, Australia. To obtain the α-KO/ActC++ mice, heterozygous α-KO mice were crossed with double heterozygous ActC++ mice. A competitive genomic PCR screening strategy with specific primers was used to confirm positive progeny.
Animals were allocated in the experimental groups as follows: Male mice WT $n = 8$, ActC++ $n = 8$, $\alpha$-KO $n = 5$, $\alpha$-KO/ActC++ $n = 4$; Female mice WT $n = 8$, ActC++ $n = 8$, $\alpha$-KO $n = 5$, $\alpha$-KO/ActC++ $n = 5$. For the stereological, serum, western blot, and bone density analyses, 8 week-old animals (WT, ActC++, $\alpha$-KO, and $\alpha$-KO/ActC++) were used. Since 100% survival rate was noted at this time point for all experimental groups. Four animals, randomly selected from each experimental group, were used for the stereological and western blot analyses. Serum analysis was conducted in three animals, randomly selected from each experimental group.

**Mouse monitoring**

Changes in body weight, tumor mass, BAR (bright, alert, and responsive) score, general clinical signs (inactive, hunched posture, coat rough-fun on end, red eye/nose discharges, pink staining of the neck, and dehydration), behavioural signs of pain, and water balance were monitored daily. Animals were sacrificed when one of the following humane end-points was observed: (i) weight loss of 10% or more over 24 h; (ii) weight loss of 20% or more plus one other clinical sign compared with the control group; and (iii) weight loss of 25% compared with the control group.

**Tissue collection**

Animals were anaesthetized and serum obtained by cardiac puncture. Animals were then euthanized by cervical dislocation. Tail biopsy was obtained and snap frozen to confirm genotype of interest. The $\alpha$-KO and $\alpha$-KO/ActC++ mice were sacrificed at 12–17 weeks of age, based on their survival rates and when clinical manifestation of any of the human end-points reported earlier was recorded. WT and ActC++ mice survived up to 30 weeks of age.

When animals were sacrificed, testis, ovary, gastrocnemius, and femur were collected. Part of the gastrocnemius was snap frozen for further analysis, the remaining part of the gastrocnemius was stored in Bouin’s solution for 4–6 h and then transferred to 70% ethanol for 12–24 h, embedded in paraffin, sectioned and mounted on Superfrost microscope slides (Menzel-Glaser).

**Stereological analysis**

Serial sections were stained with hematoxylin and eosin and photographed on an Olympus microscope (40x objective). Average fiber area in gastrocnemius muscles was calculated by counting 200–300 fibers in transverse section of muscles from four mice for each genotype and calculating the average total area of the counted fibers normalized by the number of counted fibers.\(^{21}\)

**Western blot analysis**

Frozen tissues from four animals per group were homogenized with the Tissue Lyser II (Qiagen Cat # 85300) for 5 min at 30 Hz in ice-cold RIPA Buffer [150 mM NaCl, 1% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 (Sigma-Aldrich Cat R0278)] containing protease and phosphates inhibitor (Thermo Scientific cat 78440) according to the manufacturer’s instructions, and the whole homogenate was used for further analysis. Sample protein concentration was determined using the BCA Protein Assay Reagent KIT (Thermo Scientific cat 23225), and equivalent amounts of protein from each sample (40 μg) were subjected to electrophoretic separation on 12% SDS-PAGE acrylamide gels in Tris-Glycine-SDS Running. Following electrophoresis separation, proteins were transferred to a nitrocellulose membrane (GE Healthcare cat 10600003) in Transfer Buffer (250 mM Tris, 1.92 M Glycine, 10% Methanol), blocked with Odyssey Blocking buffer (Li-Cor cat 927–40000) for 1 h, followed by an overnight incubation with primary antibody dissolved in Odyssey blocking buffer containing 0.2% Tween-20 (Sigma-Aldrich cat P2287). Antibodies used were the following: Atrogin-1 (ECM Bioscience cat AP 2041), Murf-1 (ECM Bioscience cat MP3401), Beclin-1 (Abcam cat 55878), SQSTM1/p62 (Abcam cat 91526), LC3A/B (Abcam cat 62721), Myostatin (Abcam cat 71808), Smad 2 (Abcam cat 47083), p-Smad-2 (Abcam cat 53100), Smad-3 (Abcam cat 40854), p-Smad3 (Abcam cat 51451), and GAPDH (Abcam cat 9484).

After an overnight incubation, blots were incubated with secondary antibodies diluted in Odyssey Blocking Buffer for 1 h at room temperature. Secondary antibodies used were the following: IRDye 800CW Goat Anti-Rabbit IgG, H+L (Li-corr cat 926–32211) and IRDye 680LT Goat Anti-Mouse IgG2b (Li-corr cat 926–68052).

Densitometry measurements were performed using the software Image Studio Lite v4.0 (Li-cor) by determining the signal intensity of each band after a background optimization and normalizing to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Averaged values from three independent experiments were used for the final statistic analysis.

Inflammatory cytokines were assessed using 0.5 μL of serum, electrophoresed on a 12% SDS-PAGE acrylamide gels and probed with primary and secondary antibodies following exactly the same protocol reported in the preceding paragraph.\(^{22}\) Antibodies used were as follows: IL-6 (Abcam cat 6672), IFN-γ (Abcam cat 133566) and TNF-α (Abcam cat 9739). Signal intensity of each band was normalized to the mouse IgG2b fraction.
**Bone analysis**

Skin was removed from the euthanized animal, using a scalpel to cut the cartilage between the femur and tibia; the muscle from femur and around hip joint was removed. The bone isolated was cleaned and stored at −80° in PBS soaked gauze. Femur X-Ray images were acquired using the In Vivo MS FX PRO (Bruker). X-Rays were generated at 35 KV (Filter 0.4 mm, fstop 2.80, FOV 200). Bone density was calculated according to manufacturer’s guidelines.

**Testosterone, estradiol and activin-α ELISA**

Serum levels of Testosterone and Estradiol were assessed from three males and three female per group using Abcam Elisa kits (cat 108666 and 108667), according to the manufacturer’s instructions. Serum levels of Activin A were assessed from three animals per group using the Quantikine ELISA from (R&D cat DAC008), according to the manufacturer’s instructions.

**Statistical analysis**

Statistical analysis was conducted using One-Way ANOVA, followed by Tukey’s correction for confidence intervals and significance (GraphPad Prism, version 5). For the survival analysis, the Log-rank (Mantel–Cox) test was used. Statistical significance was assumed if P was <0.05.

**Results**

**Activin-βC overexpression modulates gonadal tumours, reduces activin-A levels, and prolongs survival in the α-KO mice**

The α-KO mice developed Sertoli and granulosa cell tumours, leading to a lethal wasting syndrome characterized by progressive weight loss and death. At 8 weeks of age, there was an increase in testis (322 ± 30 mg, P < 0.0001) and ovarian weights (34 ± 1 mg, P < 0.0001) in the α-KO mice compared with WT controls (testis 107 ± 30 mg, ovary 8 ± 1).

By 8 weeks, the tumor-associated increase weight was reduced in the α-KO/ActC++ mice vs. the α-KO counterpart, with testicular (180 ± 40 mg) weight not statistically different from WT controls. (Figure 1A and 1B).

The serum levels of activin-A in the α-KO mice were significantly elevated in both males (4213 ± 50.53 vs. 134.5 ± 31.45 pg/ml) and females (4257 ± 126.8 vs. 204.5 ± 37.42 pg/ml) compared with the WT age-matched controls. However, in the α-KO/ActC++ mice, the serum levels of activin-A were reduced in both male (1392 ± 116.4 pg/ml) and female mice (3182 ± 199.5 pg/ml) compared with the α-KO mice (Figure 1C and 1D).

As shown in Figure 1E and 1F, overexpression of activin-βC in the α-KO mice increased survival in both male and female mice. At 13 weeks, 49% of male α-KO/ActC++ mice survived vs. 0% of the α-KO group (chi square 33.95, P < 0.0001) Figure 1E. At 15 weeks, 56% of female α-KO/ActC++ mice survived vs. 0% of the α-KO mice (chi square 31.22, P < 0.0001) Figure 1F.

At 12–17 weeks of age, despite a significant difference in the ovarian weights between the α-KO and α-KO/ActC++ mice, there was no significant in testicular weights between these two groups (Figure S1A and 1B), thus suggesting that activin-βC delayed but not completely abolished tumor growth.

**Activin-βC overexpression reduces elevated levels of inflammatory cytokines IL-6, IFN-γ, and TNF-α in the α-KO mice**

Elevated levels of IL-6, IFN-γ, and TNF-α were found in both male and female α-KO mice compared with the age-matched WT group. Male mice: IL-6 (3.30 ± 0.32 P < 0.01), IFN-γ (3.48 ± 0.48 P < 0.01), and TNF-α (3.52 ± 0.17 P < 0.01). Female mice IL-6 (3.81 ± 0.69 P < 0.01), IFN-γ (4.35 ± 0.56 P < 0.001), and TNF-α (8.0 ± 0.79 P < 0.001). Overexpression of activin-βC reduced the high levels of cytokines noted in the α-KO mice; in fact, no statistical significance was found in the cytokines levels of both male and female α-KO/ActC++ mice compared with the WT age-matched control, except for the IFN-γ in the female mice (82.62 ± 0.29 P < 0.01). When the cytokine levels in the α-KO/ActC++ mice were compared with the α-KO mice, statistically reduced levels were noted in all groups (Figure 2A and 2B).

**Activin-βC overexpression attenuates the ubiquitin proteasome and the autophagy–lysosomal pathway activation and abrogates the reduction in muscle cross-sectional area noted in the α-KO mice.**

In different conditions of muscle atrophy, there is an acceleration of the muscle proteolysis mediated by the proteasome ubiquitin pathway and it is demonstrable on muscle extracts.10,23,24 The catabolic factors Atrogin-1 and MuRF1 involved in the ubiquitin-proteasome pathway were assessed in the gastrocnemius of the α-KO mice in order to determine if any change was present in the α-KO/ActC++ mice. Additionally, three of the most representative markers of the autophagy-lysosomal degradation pathway were assessed: Beclin-1 as an indicator of autophagy induction, LC3A/B to measure autophagosome abundance, and p62/SQSTM1 as a marker of substrate sequestration and eventual degradation.17
As shown in Figure 3, there was a clear increase in Atrogin-1 and MuRF1 in both male (Figure 3A) (Atrogin-1 1.60 ± 0.60 \( P < 0.05 \); MuRF-1 1.90 ± 0.60 \( P < 0.01 \)) and female (Atrogin-1 2.00 ± 0.30 \( P < 0.01 \); MuRF-1 1.80 ± 0.17 \( P < 0.01 \)) \( \alpha \)-KO mice vs. WT age-matched controls (Figure 3B).

Beclin-1, an upstream regulator of autophagic sequestration,\(^{25}\) was increased in both male (2.15 ± 0.31 \( P < 0.0001 \)) and female \( \alpha \)-KO (1.48 ± 0.28 \( P < 0.05 \)) vs. the WT age-matched controls, suggesting an activation of the autophagy pathway (Figure 4A and 4B).
p62, measured as measure of substrate sequestration into autophagosomes (p62 binds LC3 and substrates marked for degradation by ubiquitinilation\textsuperscript{26}), was elevated in both male (1.28 ± 0.14 \( P < 0.01 \)) and female \( \alpha \)-KO (1.18 ± 0.11 \( P < 0.05 \)) vs. WT age-matched controls (Figure 4A and 4B).

The levels of microtubule-associated protein 1 light chain 3A/B isoform I (LC3A/B-I) was elevated in both male (2.69 ± 0.86 \( P < 0.01 \)) and female \( \alpha \)-KO (3.31 ± 0.74 \( P < 0.0001 \)) vs. WT age-matched controls, whereas no statistical differences were noted for the levels of LC3A/B-II in both male and female mice (Figure 5A and 5B).

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*Figure 2* Activin-\( \beta \)C overexpression reduces elevated levels of inflammatory cytokines IL-6, INF-\( \gamma \), and TNF-\( \alpha \) in the \( \alpha \)-KO mice. Quantification of western blots signal intensities for inflammatory cytokines IL-6, INF-\( \gamma \) and TNF-\( \alpha \) in the WT, ActC++, \( \alpha \)-KO and \( \alpha \)-KO /ActC++ mice are shown. 8 week-old males (a) and females (b) mice \( n = 4 \) per group. Normalized values are expressed as relative intensities (800 nm channel/700nm channel) to WT. Signals from IL-6, INF-\( \gamma \) and TNF-\( \alpha \) appear as green fluorophores and signals from Mouse IgG2b appear as red fluorophores. Values are mean ± SEM. ns = \( P > 0.05 \); *\( P \leq 0.05 \); **\( P \leq 0.01 \); ***\( P \leq 0.001 \); ****\( P \leq 0.0001 \).
Overexpression of activin-βC prevented the rise in Atrogin-1 and MuRF1 in both sexes. In fact, in the α-KO/ActC++ mice, except for MuRF1 in the female group, the levels of Atrogin-1 and MuRF1 were not statistically significant compared with WT age-matched controls.

Additionally, overexpression of activin-βC prevented the rise in Beclin-1 and p62 in both sexes and the rise of LC3A/B-I in the male group. The levels of Beclin-1 and p62 in the α-KO/ActC++ mice were not statistically significant compared with WT age-matched controls. The levels of LC3A/B-I were not statistically significant in the male α-KO/ActC++ group only compared with WT age-matched controls.

To explore the mechanism by which activin-βC overexpression regulates the atrophy-specific ubiquitin ligases, we assessed the levels of myostatin in order to investigate its potential contribution to the activation of the ubiquitin proteasome pathway activation, and we also assessed Smad levels across the groups analyzed. We investigated both Smad-2 and Smad-3 intracellular effectors. No statistical differences in the levels of myostatin were found in both male and female mice (Figure 6A and 6B).

Phospho Smad-2/Smad-2 was markedly increased in the α-KO mice, and overexpression of activin-βC blocked this increase in both male (Figure 7A) and female mice (Figure 7B). No statistically significant changes were noted in the phosphorylation of Smad-3 (Figure 7C and 7D); thus, suggesting that Smad-2 is the predominant intracellular pathway regulating the activin-A effect on Atrogin-1 and MuRF-1 in the gastrocnemius of the α-KO mice. In support of this, when
activin-βC was overexpressed, a clear reduction of Smad-2 phosphorylation was evident in the α-KO mice.

Additionally, histological assessment of the gastrocnemius (Figures 8 and 9) showed that the average cross-sectional area in the α-KO mice was reduced compared with the WT controls in male (−63% \( P < 0.01 \)) and female (−60% \( P < 0.01 \)) mice (Figure 10A and 10B). Overexpression of activin-βC abrogated this decline, restoring the muscle cross-sectional area to levels not statistically different compared with WT controls.

**Activin-βC overexpression has no impact on testosterone, estradiol, or bone density in the α-KO mice**

A relationship between inflammation and bone disease has been established in a variety of models of inflammatory disease, as well as human studies.\(^{27–29}\) Therefore, we assessed the impact of elevated inflammatory cytokines in the α-KO mice on bone density. Femora from WT, ActC++, α-KO, and...
α-KO/ActC++ mice were analyzed. A reduction of bone density was noted in both male (0.33 ± 0.01 g/cm³, \(P < 0.0001\)) and female (0.33 ± 0.02 g/cm³, \(P < 0.02\)) α-KO mice compared with the WT control animals. No difference was noted between the α-KO and α-KO/ActC++ in both male and female mice (Figure 10C and 10D).

Testosterone and estradiol serum levels were also assessed in male (Figure 10E) and female (Figure 10F)
mice to determine their potential contribution in the changes in bone density of the \( \alpha \)-KO and \( \alpha \)-KO/ActC++ groups compared with the WT controls. Results showed no statistically significant changes in both testosterone and estradiol serum levels between the \( \alpha \)-KO and \( \alpha \)-KO/ActC++ mice.

Discussion

Although cachexia represents a debilitating syndrome affecting the majority of cancer patients and is negatively correlated with the patient’s quality of life, the biological mechanism leading to this multifaceted syndrome is poorly understood. A deeper understanding of the pathophysiology of cachexia and its progression is needed in order to lead to development of new therapies, as cachexia is usually considered a causative factor of cancer patient mortality and morbidity.\(^1\)

Given the urgent need for an effective treatment to combat cachexia, animal models have an essential role not only for assessing the efficacy and safety of a potential treatment but also to better understand biological mechanisms involved in muscle wasting and cancer cachexia.\(^3\) The inhibin-\( \alpha \) deficient (\( \alpha \)-KO) mouse model used in this study was first described by Matzuk et al. and it clearly showed the importance of activins: members of the TGF-\( \beta \) family ligands, in the progression of cancer cachexia.\(^7\) After genetic removal of the inhibin-\( \alpha \) subunit, these mice develop gonadal sex-cord stromal tumours within 6 weeks, causing death in males and females in 12 and 17 weeks, respectively. The development of gonadal cancers is followed by a cancer cachexia-like wasting syndrome that is associated with weight loss and stomach and liver related problems.\(^7\) The gonadal tumours secrete high levels of activin-A and inflammatory cytokines, which are responsible for the pronounced effect on weight loss.

Zhou et al. reported the importance of the activin signalling pathway mediated by the activin type-2 receptor (ActRIIB) to reverse cachexia and showed that using a decoy receptor not only abrogates the wasting process in skeletal muscle and heart but also increases survival. Therefore, activins and possibly other members of the TGF-\( \beta \) superfamily clearly have a central role in the pathophysiology of cancer cachexia with the activin/myostatin signalling pathway being identified as a suitable candidate to reverse cancer-associated cachexia in murine models.\(^10\)

A study conducted by our group described for the first time a new activin-A antagonist: activin-\( \beta \)C. This protein acts as an activin-A antagonist both \textit{in vitro} and \textit{in vivo}.\(^18\) Based on this evidence, we used mice overexpressing activin-\( \beta \)C crossed with \( \alpha \)-KO mice to assess the biological effect of activin-\( \beta \)C overexpression on the onset of gonadal tumors and cancer-associated cachexia. We did find that the \( \alpha \)-KO/ActC++ mice had no significant weight loss, increased survival, and that the gonadal tumor formation was modified.\(^19\) Building on our previous study, we wanted to characterize the biological mechanism by which the \( \alpha \)-KO /ActC++ showed increased survival, assessing for the first time the effect of overexpression of activin-\( \beta \)C on muscle degradation, bone density, and inflammatory and hormonal profile.

Figure 6 Activin-\( \beta \)C overexpression does not affect the myostatin levels in the \( \alpha \)-KO mice. Quantification of western blots signal intensities for myostatin in 8 week-old males (c) and females (d) mice \( n = 4 \) per group. Normalized values are expressed as relative intensities (800 nm channel/700 nm channel) to WT. Signals from Myostatin appear as green fluorophores, and signals from GAPDH appear as red fluorophores. Values are mean ± SEM. ns = \( P > 0.05 \); * \( P \leq 0.05 \).
Our findings revealed a previously unappreciated function for activin-βC on muscle metabolism and activation of transcription factors involved in muscle protein degradation. We defined the molecular mechanism by which activin-βC increases survival and ameliorates the muscle wasting phenotype in the α-KO mice. We confirmed that overexpression of activin-βC reduced serum activin-A and it also reduced inflammatory cytokines, factors recognized to have an essential role in the progression of cancer-associated cachexia and directly involved in triggering the mechanism of wasting. Despite a significant reduction in the serum levels of activin-A in both male and female α-KO/ActC++ mice vs. the α-KO counterpart, the current study revealed that this reduction is less marked in the female mice. Interestingly, this trend reflects the tumor growth data (Figure 1A and 1B). In fact, gonadal weight in the males α-KO/ActC++ was not statistically significant vs. WT mice, while in the female α-KO/ActC++, a statistically significant change was still present vs. the WT counterpart.

It has been hypothesized that the gonadal tumors developed in the α-KO mice are responsible for the secretion of high levels of activin-A and inflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), and interferon-gamma (IFN-γ). Our data support this hypothesis and show that the modulation of gonadal cancers by overexpressing activin-βC also has a previously unappreciated

![Figure 7 Activin-βC overexpression reduces phosphorylated Smad2, but not Smad3 in the α-KO mice. Quantification of western blots signal intensities for Smad-2/p-Smad2 and Smad-3/p-Smad-3 in the gastrocnemius of male (a, c) and female (b, d) mice, 8 week-old mice n = 4 per group. Normalized values are expressed as relative intensities (800 nm channel/700 nm channel) to WT. Signals from Smad-2/p-Smad-2, Smad-3 and p-Smad3 appear as green fluorophores, and signals from GAPDH appear as red fluorophores. Values are mean ± SEM. ns = P > 0.05; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001.](image-url)
impact on inflammatory cytokines, resulting in a significant biological effect. Based on our previous study in which we showed that activin-βC is an activin/myostatin signalling pathway antagonist at the receptor level, we conclude that the effect of activin-βC on cancer-cachexia is likely to act via a two-fold mechanism: modulation of gonadal tumor growth and antagonistic effect on the signaling pathway activated by TGF-β ligands myostatin and activin-A (previously showed to be aberrant in cancer cachexia) in gonadal and extra-gonadal tissues like skeletal muscle.

Reduced levels of Atrogin-1 and MuRF-1 were evident in the α-KO/ActC++ compared with the α-KO mice. Both myostatin and activin bind to the ActRIIB to initiate a signalling cascade leading to an increased expression of Atrogin-1 and MuRF-1, resulting in increased degradation of myofibrilar proteins through the ubiquitin-proteasome pathway. In our previous studies, we showed that activin-βC could antagonize the activin signaling pathway both in vivo and in vitro, not only forming a dimer complex activin-AC and therefore reducing the bioactive levels of activin-A but also binding to the ActRIIA and IIB and acting as a receptor antagonist in vitro. These data support the findings described in the current study; in fact, overexpression of activin-βC attenuated the ubiquitin-proteasome system, decreasing the expression of both Atrogin-1 and MuRF-1.

We also showed that the expression of the autophagic-lysosomal pathway associated effectors Beclin-1, p62, and LC3A/B-I was increased in the α-KO mice, thus suggesting an increased proteolytic activity that contributes to the loss of muscle protein in cancer cachexia.

Overexpression of activin-βC attenuated this activation, decreasing the expression of Beclin-1 and p-62 in the α-KO/ActC++ mice vs. the α-KO counterpart. However, overexpression of activin-βC did not reduce the protein levels of LC3A/B-I in the α-KO/ActC++ mice vs. the α-KO counterpart.

These data suggest an activation of the autophagic pathway in the α-KO mouse model of cancer cachexia and support previous evidence suggesting that autophagy significantly contributes to muscle wasting, thus suggesting the autophagic-proteolytic system as a possible target for therapeutic strategies.

Additionally, in the α-KO/ActC++ mice, the Smad2 phosphorylation and therefore activation was reduced compared with the α-KO animals. This has important biological consequences for protein degradation in muscles, as Smad-2

Figure 8 Hematoxylin and eosin staining of gastrocnemius fibers in a transverse section from WT (a), ActC++ (b), α-KO (c) and α-KO/ActC++ (d) male mice. Scale bar 100 μM, 20× objective.
phosphorylation stimulates activation of FOXO3a, which is then de-phosphorylated and moves to the nucleus, where it activates the expression of both Atrogin-1 and MuRF-1.11 A beneficial effect of activin-βC was also noted in the gastrocnemius tissue, with α-KO/ActC++ mice showing less morphometric signs of muscle atrophy.

Inflammation of almost any cause is usually associated with bone loss.32 It has been shown that some inflammatory cytokines have a negative effect on bone formation, inducing osteoclast formation. TNF-α, for example, plays a critical role in enhancing osteoclast-mediated bone resorption by interacting with the receptor activator of nuclear factor-kB ligand.33 IL-6 is another important inflammatory cytokine, which has a negative impact on the bone metabolism supporting osteoclasts via the interaction with mesenchymal cells.34,35 Hormonal alterations themselves play an important role for both skeletal development and maintenance. Androgens and estrogens, for example, have been shown to have central roles for bone homeostasis in men and rodents.36 Previous studies have shown that the effects of androgens on the bone metabolism may be mediated by the androgen receptor or via the aromatization of androgen to estrogens.37

Additionally, inhibin-α has an impact on the bone metabolism. It has previously been hypothesized that continuous exposure to inhibin is anabolic to the skeleton. However, lack of inhibin leads to an acceleration of osteoblast and osteoclast development, leading to an increase in bone turnover.38 Therefore, with the present study, we investigated if changes in the inflammatory cytokines and hormonal alterations had any effect on bone density and whether overexpression of activin-βC was associated with this effect. No difference was noted between the WT and ActC++ groups; however, both the α-KO and α-KO/ActC++ mice had reduced bone density compared with the WT age-matched controls. Serum levels of testosterone and estradiol did not differ in the α-KO and α-KO/ActC++ groups; therefore, the reduced bone density observed in these mice is likely to be a consequence of the high levels of inflammatory cytokines and lack of inhibin-α. We speculate that the reduced bone density noted in α-KO/ActC++ compared with the α-KO mice, despite a reduction in inflammatory cytokines reported in the α-KO/ActC++ mice, is caused by the lack of inhibin-α, as the hormonal profile did not show significant changes. These findings, taken together, suggest that activin-βC does not play an independent central role in the regulation of bone metabolism.
Figure 10  Activin-βC overexpression reverses fiber atrophy and does not affect the bone density or the hormonal profile in the α-KO mice. Morphometric analysis of the gastrocnemius showing the gastrocnemius average fiber area (μm²) from male (a) and female (b) 8 week-old mice, n = 4 per group. Bone density (g/cm³) in male (c) and female (d) mice. Eight week-old mice, n = 4–8 per group. Serum testosterone and estradiol levels in male (e) and female (f) mice. 8 week-old mice, n = 3 per group. Values are mean ± SEM. ns = P > 0.05; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001.
Conclusion

This study shows the importance of activin signaling in cancer-associated cachexia, providing further rationale for the therapeutic potential of anti-activin therapies. The data support the importance of activin-βC as a regulator of activin-A bioactivity in vivo. We showed for the first time that activin-βC has an effect in modulating the catabolic factors, Atrogin-1 and MuRF-1, which are responsible for triggering the mechanism of wasting not only in cancer-associated cachexia but also in many other catabolic conditions. Additionally, we showed that activin-βC has an effect in modulating two of the most important markers associated with the autophagic-lysosomal pathway, which has been suggested to contribute to muscle wasting in different experimental conditions, such as denervation, starvation, and sepsis.

Overexpression of activin-βC in the α-KO mouse model of cancer-associated cachexia showed a biological effect, reducing circulating levels of activin-A, inflammatory cytokines involved in the mechanism of wasting and ameliorating the muscle loss. Further studies are necessary in order to clarify if the effect of activin-βC is likely to be anabolic only when deregulation of activin signalling is present, or if its use might be beneficial in conditions such as muscoskeletal disorders not directly related to the activin signalling pathway.

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Conflict of interest

The authors declare there is no conflict of interest relating to this work.

Supporting information

Supporting information may be found in the online version of this article.

Figure S1. Overexpression of activin-βC modulates ovarian but not testicular weight when an advanced stage of tumor development is present. Male (a) and female (b) gonadal weight in 12–17 week-old mice. Values shown represent mean ± SEM. ns = p > 0.05; ** p > 0.01; ****p > 0.0001.

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