Biological Sex Differences of Fibrosis During the Development of Cancer Cachexia

Tyrone Washington[1], Eleanor R. Schrems[1], Wesley S. Haynie[1], Megan Rosa-Caldwell[1], Jacob Brown[1], Landen Saling[1], Seongkyun Lim[1], Richard Perry[1], Lemuel A. Brown[1], David Lee[1], and Nicholas Greene[1]

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January 4, 2023

Abstract

AIM Cachexia is characterized by losses in lean body mass and its progression results in worsened quality of life and exacerbated outcomes in cancer patients. However, the role and impact of fibrosis during the early stages and development of cachexia in under-investigated. The purpose of this study was to determine if fibrosis occurs during cachexia development, and to evaluate this in both sexes. **Methods** Female and male C57BL6/J mice were injected with PBS or Lewis Lung Carcinoma (LLC) at 8-week of age and tumors were allowed to develop for 1, 2, 3, or 4 weeks. 3wk and 4wk female tumor-bearing mice displayed a dichotomy in tumor growth and were reassigned to high tumor (HT) and low tumor (LT) groups. *In vitro* analyses were also performed on co-cultured C2C12 and 3T3 exposed to LLC conditioned media. Immunohistochemistry and quantitative PCR analysis were used to investigate fibrosis and fibrosis related signaling in skeletal muscle. **Results** Collagen deposition in skeletal muscle was increased in the 1wk, LT and HT groups in female mice. However, collagen deposition was only increased in the 4wk group in male mice. In general female mice displayed earlier alterations in ECM related genes beginning at 1 wk post-LLC injection. Whereas this was not seen in males. **Conclusions** While overall tumor burden is tightly correlated to cachexia development in both sexes, fibrotic development is not. Male mice did not exhibit early-stage alterations in ECM related genes contrary to what was noted in female mice.

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Running Title: Fibrosis and Cancer Cachexia Development

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Abstract

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Methods

Female and male C57BL6/J mice were injected with PBS or Lewis Lung Carcinoma (LLC) at 8-week of age and tumors were allowed to develop for 1, 2, 3, or 4 weeks. 3wk and 4wk female tumor-bearing mice displayed a dichotomy in tumor growth and were reassigned to high tumor (HT) and low tumor (LT) groups. *In vitro* analyses were also performed on co-cultured C2C12 and 3T3 exposed to LLC conditioned media. Immunohistochemistry and quantitative PCR analysis were used to investigate fibrosis and fibrosis related signaling in skeletal muscle.

Results

Collagen deposition in skeletal muscle was increased in the 1wk, LT and HT groups in female mice. However, collagen deposition was only increased in the 4wk group in male mice. In general female mice displayed earlier alterations in ECM related genes beginning at 1 wk post-LLC injection. Whereas this was not seen in males.

Conclusions

While overall tumor burden is tightly correlated to cachexia development in both sexes, fibrotic development is not. Male mice did not exhibit early-stage alterations in ECM related genes contrary to what was noted in female mice.

Significance statement

Cachexia is the immediate cause of death for 20-40% of cancer patients. Cachexia is characterized by tissue fibrosis. The impact of cachexia progression on skeletal muscle fibrosis is unknown. Additionally, how sex alters the cachexia-associated fibrotic response in skeletal muscle is unknown. The principal finding of this study was that while tumor burden is tightly correlated to cachexia progression in both sexes, fibrotic development was not. Females demonstrated mild collagen deposition early whereas males did not demonstrate collagen deposition until much later with a much more exaggerated response. The lack of SMAD 2 and 3 induction suggests a non-canonical signaling pathway is involved in ECM regulation during the progression of cachexia.

Introduction

Cancer cachexia is a progressive and involuntary wasting condition of lean body mass that afflicts approximately 80 % of all cancer patients and can be attributed to 20-40% of cancer-associated deaths[1, 2]. Cancer cachexia is defined as losses in body mass >5 %, primarily due to loss of muscle mass which can be accompanied with or without losses in adipose tissue[3, 4]. To date, no singular method of treatment has shown effective at eliminating cancer cachexia development due to the systemic and complex nature of its underlying mechanisms[5-7]. Further complicating research into effective treatments for cancer cachexia is the emerging role of sexual dimorphism on cancer cachexia progression, as recent studies have shown that males and females can differentially respond to cancer cachexia[8-10]. Metabolic and inflammatory instabilities are known contributors to cancer cachexia progression and have been greatly examined [11, 12], however the role of fibrosis on the development of cancer cachexia remains incompletely understood.

Excessive fibrosis of healthy skeletal muscle worsens overall outcomes in cancer patients [13]. Moreover, unlike metabolic and inflammatory abnormalities that can improve following cancer cessation, this fibrosis of vital and functional lean tissue is largely irreversible and can affect patients throughout their lifespan. The extracellular matrix (ECM) is composed primarily of collagen 1 and 3[14]. The ECM is critical for force transduction, growth factor secretion, etc. The matrix metalloproteinases (MMPs) are responsible for the degradation of collagens, and in skeletal muscle MMP2 and MMP9 are typically the most abundant[15]. Skeletal muscle MMP expression is altered in cancer cachexia[16] which may contribute to the excessive fibrosis associated with cancer cachexia. Transforming growth factor-β (TGF-β) is a transcriptional activator of many cellular processes including the activation of satellite cells as well as synthesis, degradation, and remodeling of the ECM that surrounds skeletal muscle[17, 18]. Upregulation of TGF-β can result in excessive ECM dysregulation and deposition of ECM components ultimately resulting in the replacement of healthy skeletal muscle with non-contractile proteins such as collagens [19, 20]. This upregulation of TGF-β can be induced via its upstream regulator in Interleukin-6 (IL-6), an inflammatory cytokine which is commonly elevated in cachectic cancer patients [21]. Taken together, these data suggest fibrosis in cancer patients is altered via TGF-β, and evaluation of TGF-β and its downstream effectors could provide insights on fibrosis as it pertains to cancer cachexia progression.

Males and females exhibit sexual dimorphism in skeletal muscle properties such as fiber type composition within the same muscle, mitochondrial content, and number of satellite cells innately, and this dimorphism is documented in some muscle conditions such as disuse atrophy [22-24]. Differences in the relative amounts of circulating hormones like testosterone and estrogen only adds to the complexity of the male and female phenotypic makeup within skeletal muscle. For example, testosterone is typically higher in males and promotes muscle protein synthesis and regeneration, while estrogen is typically higher in females and has a greater impact on reducing inflammation [25, 26]. Therefore, female mice could possess a greater protective effect from inflammatory-based muscle wasting conditions such as cancer cachexia when compared to males[10]. Innate differences between sexes can therefore greatly affect overall skeletal muscle health and even skeletal muscle health in pathologies such as cancer cachexia [8, 10].

This study is, to our knowledge, the first to evaluate the role of fibrosis during the development of cancer cachexia across both sexes. Implantation of Lewis Lung Carcinoma (LLC) cells allows for the controlled development of cancer cachexia, and thorough analysis of fibrotic pathways during the progression of cachexia. Evaluation of these fibrotic markers could elucidate potential avenues for therapeutic interventions aimed towards the prevention of fibrosis and its contributions to cancer cachexia development. Therefore, the purpose of this study was to utilize the LLC allograft model of cachexia to evaluate key regulators of the ECM during the controlled development of cancer cachexia. We hypothesized the ECM and signaling pathways involved in its modulation would be altered prior to cachexia. Herein we provide novel evidence of altered fibrotic signaling coinciding with cancer cachexia development, however this varied between sexes. These data highlight the emerging role of fibrosis on cancer cachexia and further strengthens the growing need for research in both males and females as sex continues to create divergent responses to cancer cachexia and its underlying mechanisms.

Methods

Animals and Interventions

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas, Fayetteville. Male and female C57BL/6J mice were purchased from Jackson Laboratories. We have previously reported on the phenotype and aspects of mitochondrial and protein metabolism in these animals[27-29]. The mice were kept on a 12:12 h light–dark cycle with *ad libitum* access to normal rodent chow and water. LLC cells (1×10^6) suspended in 100 µL sterile PBS were implanted to the hind flank of mice at 8 weeks of age as previously described [28]. The tumor was then allowed to develop for 1, 2, 3, or 4 weeks in separate cohorts. For sham control, one group of mice received a bolus injection of 100 µL sterile PBS. PBS controls were age-matched to the most cachectic group (4 weeks post-implantation, 12 weeks of age at tissue collection). In the female mice, there was a clear dichotomization between the 3 and 4 weeks groups to cluster into smaller or larger tumor sizes. These 2 groups were then isolated and split into low tumor (LT; [?] 1.2 g) and high tumor (HT; [?] 2 g) groups based on this dichotomization of tumor size as described previously[27]. Animal tissues, organs, and blood plasma were quickly collected under isoflurane anesthesia prior to euthanasia. Tissues were weighed and snap-frozen in liquid nitrogen for further processing and stored at -80°C. The tibialis anterior muscle from males (TA) and plantaris muscle from females were submerged in optimum cutting temperature compound (OCT) and then placed in liquid nitrogen cooled isopentane. OCT mounted tissue was then stored at -80°C for histological analysis.

Lewis Lung Carcinoma and Implantation

LLC cells were prepared as described previously [28]. LLC cells (ATCC CRL-1642) were plated at passage 2. Cells were cultured in 250 ml culture flasks in DMEM supplemented with 10% fetal bovine serum supplemented with 1% penicillin and streptomycin. Once cells reached confluence, they were counted, trypsinized and diluted in PBS for implantation. Mice were anesthetized with isoflurane and hair was removed from the right hind flank. LLC cells ($1x10^6$) suspended in 100 µl sterile PBS and injected subcutaneously into the hind flank of mice at 8 weeks of age as previously described[28]. Tumors developed for up to 4 weeks. Experimental endpoints were adjusted for signs of distress and veterinary recommendation for humane care.

Myoblast and Fibroblast Co-Culture

C2C12 myoblast cells (ATCC CRL-1772) and 3T3 fibroblast cells (ATCC CRL-1658) were co-cultured for an *in vitro* analysis of fibroblasts to skeletal muscle interaction. C2C12 cells were grown until they reached ˜80% confluency at passage 2 where they were then transferred to a 6-well plate and differentiated for 5 days. 3T3 fibroblast cells were then added via a transwell-insert, which possesses a semi-permeable membrane, into the 6-well plate. LLC conditioned media (LCM) or control media (Veh) were added for 24hr. Both myotubes and 3T3 fibroblasts cells were harvested for subsequent RT-qPCR and Western Blot analysis.

RNA Isolation, cDNA synthesis, and Quantitative Real-Time PCR

RNA isolation, cDNA synthesis, and quantitative real-time PCR were performed as we have previously described [29, 30]. All targets were assayed using Taqman probes including: 18S (Mm03928990_g1), Collagen 1 (Mm00801666_g1), Collagen 3 (Mm00802305_g1), MMP-2 (Mm00439498_m1), MMP-9 (Mm00439498_m1), Timp-1 (Mm01341361_m1), Timp-2 (Mm00441825_m1), TGF-β1 (Mm01178820_m1), SMAD 2 (Mm00487530_m1), SMAD 3 (Mm01170760_m1). Taqman probes were purchased from Applied Biosystems. RT-qPCR measured cycle threshold (Ct) and the ΔCt value was calculated as the difference between the Ct value and the 18S Ct value. Final quantification of mRNA abundance was calculated using the ΔΔCT method Ct = [ΔCt (calibrator) – ΔCt (sample)]. Relative quantifications were then calculated as $2^{-\Delta\Delta\hat{}\tau}$. 18S Ct values were confirmed to not differ between experimental conditions for any comparison.

Histology

TA and plantaris skeletal muscle stored in OCT were cryosectioned into 10μm thick sections on polarized microscope slides. Muscle sections were then histologically stained with Picro Sirius Red for quantification and analysis of overall collagen content within the muscle. Images were analyzed using Nikon NIS Elements BR software package.

Statistical Analysis

Normality of data was established via a Shapiro Wilk test prior to any parametric analysis. A one-way ANOVA was employed as the global analysis for each dependent variable in both experiments. All statistics were performed across experimental groups within each sex. Where significance was detected, differences among means were determined by a Student–Newman–Keuls post hoc test. A Student's t-test was utilized for the myotube diameter experiment. For all other cell culture experiments, a two-way ANOVA was employed to test for main effects of treatment (Veh or LCM) and cell type (C2C12 or 3T3) and whether any interactions existed between the dependent variables. For all experiments, statistical significance was set a P [?] 0.05. All data were analyzed using the Statistical Analysis System (SAS) and figures were compiled using GraphPad Prism and data expressed as mean ± standard error of the mean (SEM).

Results

Confirmation of Cachexia Development.

Female and male body weight, tumor weight, hindlimb skeletal muscle weight, and organ weights of PBS and LLC-injected C57BL/6J mice were reported previously by Lim et. al. [27] and Brown et. al. [28, 29], respectively. Neither male nor female mice saw significant reductions in tumor-free body weight compared to PBS control. In the male, the HT group showed lower muscle and fat mass compared to PBS control. In female mice, the 4 wk group showed lower muscle and fat mass compared to PBS control. Additionally, in females, the LT and HT groups as well as the 3 wk and 4 wk groups in males showed splenomegaly compared to PBS control.

Excessive Deposition of Collagen in Skeletal Muscle

Picrosirius Red staining—a marker of collagen deposition—within the plantaris (female) and TA (male) muscle was performed to determine overall fibrosis during the development of cancer cachexia. 1wk and LT and HT female mice saw an ˜2-fold greater ($p<0.05$) collagen deposition compared to the PBS and 2wk groups (Figure 1A). Male mice saw no significant differences in collagen deposition until 4 weeks post-LLC injection where there was an ˜3-fold greater ($p<0.05$) deposition compared to all other groups (Figure 1B).

Collagen mRNA Abundance is Altered during Cancer Cachexia Development

In female mice, collagen 1 and 3 mRNA abundance were ˜ 2.8 and ˜1.7-fold greater in the 1 wk post-LLC injection group compared to all other groups, respectively ($p<0.05$; Figure 2A & 2B), however the ratio of Collagen 3:1 mRNA abundance was ˜ 78% lower in 1wk post-LLC injection female mice compared to PBS ($p<0.05$; Figure 2C). In male mice, the 2 wk post-LLC injection group had an ˜ 2.2-fold and 2.8-fold higher collagen 1 mRNA abundance compared to the PBS group and 4 wk post-LLC injection groups, respectively ($p<0.05$; Figure 2D). In male mice, the 3 and 4 wk post-LLC injection groups had ˜56% lower collagen 3 mRNA abundance compared to the 1 wk post-LLC injection group ($p<0.05$; Figure 2E). In male mice, the ratio of collagen 3:1 was ˜113% higher in the 4 wk post-LLC group compared to the 1, 2, and 3 wk post-LLC injection groups ($p<0.05$; Figure 2F).

Regulators of ECM Remodeling are Altered during Cachexia Development

In female mice, MMP-2 mRNA abundance was ˜1.1-fold higher in the 1 wk post-LLC injection group compared to the LT or HT groups ($p<0.05$; Figure 3A). MMP-9 mRNA abundance was ˜2.0-3.8-fold higher in the HT group compared to all the other groups ($p<0.05$; Figure 3B). Male mice saw no changes in MMP-2 mRNA abundance with LLC-injection ($p>0.05$; 3C); however, the 4 wk post-LLC injection group had an ˜9.6-fold higher MMP-9 mRNA abundance compared to all other groups ($p<0.05$; Figure 3D).

ΤΓΦ-β-ρεγυλατεδ Φιβροσις δυρινγ ἀνζερ ἀζηεξια Δεελοπμεντ

In female mice, TGF-β1 mRNA abundance was ˜2.3-fold higher in the 1wk post-LLC injection group compared to all other groups ($p<0.05$; Figure 4A). We found no differences in SMAD 2 and 3 mRNA abundance ($p>0.05$; Figure 4B & 4C). In male mice, there was an ˜1.6-fold higher TGF-β1 mRNA abundance in the 4 wk post-LLC injection group compared to the PBS and 1 wk post-LLC groups ($p<0.05$; Figure 4D). There were no differences detected in mRNA abundance of SMAD 3 ($p>0.05$; Figure 4E); however, the 4 wk post-LLC group had a ˜0.5-fold higher SMAD 2 mRNA abundance compared to the 1 wk post-LLC injection groups ($p<0.05$; Figure 4G).

In vitro Analysis of Skeletal Muscle-ECM Dynamics in a Cachectic Environment

Myotube diameter was ˜20% lower following treatment with LCM compared to Vehicle ($p<0.05$; Figure 5A & 5B). There was a main effect of myotubes to have ˜4-fold less Collagen 1 mRNA abundance compared to 3T3 fibroblasts cells ($p<0.05$; Figure 6A). There was main effect of LCM treatment to reduce Collagen 1 mRNA abundance independent of cell type ($p<0.05$; Figure 6A). There was a main effect of 3T3 fibroblasts cells to have lower Collagen 3 mRNA abundance to C2C12 myotubes independent of treatment ($p<0.05$; Figure 6B). There were main effects for myotubes to have a greater Collagen 3:1 ratio independent of treatment ($p<0.05$: Figure 6C). Additionally, there was a man effect for groups treated with LCM to have a higher Collagen 3:1 ratio ($p<0.05$; Figure 6C). There were no differences in mRNA abundance of MMP-2 in either myotubes or 3T3 cells ($p>0.05$; Figure 6D), however mRNA abundance of MMP-9 was ˜450-fold greater in 3T3 cells than in myotubes and LCM treatment reduced MMP-9 mRNA ˜200-fold in 3T3 cells compared to Vehicle ($p<0.05$; Figure 6E). There was a main effect for 3T3 cells to have lower mRNA abundance of TIMP-1 independent of treatment ($p<0.05$; Figure 6F). No differences were observed in C2C12 or 3T3 cells for protein content of β-catenin ($p>0.05$; Supplemental Figure 1C).

Discussion

Herein, we are among the first to evaluate fibrosis and associated markers during the early stages of development of cancer cachexia, specifically with regards to dimorphisms across biological sex. Our analysis consisted of measuring major ECM components such as collagens as well as modulators of the ECM (i.e., MMPs). We further examined a key signaling pathway (i.e., TGF-β/SMAD) associated with fibrosis and its downstream effectors. These key markers of fibrosis were analyzed*in vivo* and *in vitro* to create a comprehensive approach to understanding if the ECM is altered during the development of cancer cachexia, and if so, what are the likely contributors. While no direct statistical comparisons were made between males and females, we observed clear differences in fibrosis progression during the development of cancer cachexia across sex. Female mice exhibited alterations in collagen content, as well as mRNA of collagen and TGF-β as early as 1-week post-LLC injection. Male mice on the other hand displayed no significant changes in fibrosis or fibrosis-related markers until 4-weeks post-LLC injection. Specifically, the collagen deposition in females was mild compared to males (5% vs. 15%). *In vitro*co-culturing of myotubes and fibroblasts in a cachectic environment yielded interesting findings, albeit in the absence of sex distinctions, but with the added distinction of isolating muscle and ECM associated cell lines. Of note, both C2C12 and 3T3L1 cells had altered mRNA abundance in certain collagens, MMPs, and TIMP-1 relative to one another. Addition

of LCM did indeed reduce myotube diameter significantly as well as reduce Collagen 1 mRNA and increased Collagen 3:1 mRNA in both cell types. Our findings support the growing theme across the literature for divergent responses to myopathologies between male and female sexes as well as reinforce the need for these sex-based comparisons in future studies [8, 31, 32].

As we reported [27], the dichotomization of tumor burden observed in the female mice supports the correlation of overall tumor burden to the development of a cachectic phenotype. However, with regards to incidence of fibrosis, we observed increased collagen content and ECM dysregulation as early as 1wk post-LLC injection in female mice. This observation indicates that while overall tumor burden is tightly correlated to cancer cachexia in both sexes, the fibrotic development as it pertains to cancer cachexia is not as tightly correlated to overall tumor burden. In contrast, we did not observe the alterations in male mice 1-week post-LLC injection seen in female mice suggesting fibrotic development is not correlated to overall tumor burden in male mice. Male mice did not display the same early stages alterations in fibrosis markers. These data suggest both males and females experience alterations in their ECM as early as 1wk post-injection with LLC cells, but not with complete consistency across the associated regulators in MMPs and TGF-β. Recent studies report reductions in TGF-β mRNA in cardiac muscle in a colon adenocarcinoma cells 26 model of cancer cachexia [33] and increased TGF-β mRNA in adipose and skeletal muscle tissue in models of pancreatic cancer cachexia[13, 34], but all studies exhibit increased fibrosis. TGF-β then appears to be a clear and consistent regulator of fibrosis in cancer cachexia, and our findings suggest its role can even precede development, at least in female mice.

The role of TGF-β in fibrosis across pathologies has been well documented [35-37]. The canonical pathway of TGF-β is to activate its downstream regulators in SMAD 2 and 3, which in turn can induce expression of collagens including the Collagen 1 and 3 which are highly expressed in the muscle belly [36]. Dysregulations in TGF-β lead to improper deposition of ECM components via SMAD 2 and 3, ultimately resulting in fibrosis. However, here we report no significant alterations in SMAD 2 or 3 mRNA content in either male or female mice or even *in vitro* following addition of LCM. Further examination by measurement of β-catenin content is needed as crosstalk between TGF-β and the Wnt/β-catenin pathway has been noted in previous literature [38-41]. The lack of SMAD 2 and 3 induction appears to suggest a non-canonical pathway is involved in regulation of the ECM and its components in this model of cancer cachexia. However, what pathway(s) is/are involved remains unclear and requires further study.

The remodeling of the ECM is one of the most important features of cancer progression. Therefore, understanding how fibroblasts respond to a cachectic environment as well as how they may influence skeletal muscle is important to understand. Therefore, we co-cultured C2C12 myotubes with 3T3L1 fibroblasts and then created a cachectic environment via addition of LCM. We then analyzed the same key components and regulators of the ECM and fibrotic pathways that were targeted in the*in vivo* approach. We demonstrated a reduction in myotube diameter with the addition of LCM media suggesting a cachectic phenotype. We observed fibroblasts exposed to a cachectic environment reduced their gene expression of MMP-9 significantly. As collagens are a major target of MMP-9, reduced expression could lead to increased collagen deposition. This was a phenotype observed in the *in vivo*experiments. Additionally, we observed that myotubes co-cultured with fibroblasts and exposed to this cachectic environment altered their expression of collagen 3 relative to collagen 1. This suggests the cachectic environment not only induces fibroblasts to alter expression of MMP-9 but influences skeletal muscle cells to alter their expression of collagens. These findings could explain the increased collagen deposition noted in this study and the ECM remodeling noted in several other studies during cachexia.

In summary, this study set out to obtain a better understanding of fibrosis during development of cancer cachexia across biological sex. Our findings support the recent findings that females and males to display divergent responses to pathologies with females being somewhat protected from cancer cachexia [10]. While both sexes displayed increased fibrosis following the onset of canonical cachexia, females exhibited alterations in markers associated with fibrosis and ECM regulation as early as 1wk post-injection with LLC. With the noted protection females exhibit to cachexia these alterations could point to a compensatory re-

sponse. Overall, increased fibrosis is involved with the development of cancer cachexia and males and females seemingly differ in the timing of alterations in the relevant fibrosis-related markers. However, more research is necessary to ascertain the molecular regulators involved in the elevated collagen deposition and fibrosis shown in our results as TGF-β was elevated but the traditional downstream effectors in SMADs 2 and 3 and β-catenin were unchanged. A greater understanding of these pathways involved with fibrosis development during the pre- and early-stage development of cancer cachexia could point to potential therapeutic avenues and improve overall patient outcomes.

Acknowledgements

The authors thank all the various faculty, staff, and students of the Exercise Science Research Center at the University of Arkansas for their support and contributions herein.

Grants

This study was funded by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health, Awards R15AR069913/AR/NIAMS and R01AR075794-01A1/AR/NIAMS. Support has also been provided in part by the Arkansas Biosciences Institute, the major research component of the Arkansas Tobacco Settlement Proceeds Act of 2000.

Disclosures

No conflict of Interest, financial or otherwise, are declared by the authors.

Figure Legends

Figure 1 . Collagen deposition within skeletal muscle measured via Picrosirius Red staining during the progression of cancer cachexia in the A) plantaris (female) and B) tibialis anterior muscle (male).*N* of 4-5 per group. Different letters denote statistical significance at an alpha set a P [?] 0.05. 1wk, 2wk, 3wk, 4wk, denotes 1, 2, 3, and 4 weeks of cancer progression, respectively; LT and HT denote low tumor and high tumor, respectively; PBS, phosphate-buffered saline. Different letters denote statistical significance at an alpha set a P [?] 0.05.

Figure 2 . Collagen 1 and 3 mRNA abundance during the progression of cancer cachexia in females (left) and males (right). (A-B) Collagen 1 and 3 mRNA abundance in female tibialis anterior muscle. (C) Collagen 3:1 mRNA abundance female tibialis anterior muscle. (D-E) Collagen I and III mRNA abundance in male tibialis anterior muscle. (F) Collagen 3:1 mRNA abundance male tibialis anterior muscle. For females, *N* of 10-14 per group were used. For males, *N*of 7-8 per group were used. 1wk, 2wk, 3wk, 4wk, denotes 1, 2, 3, and 4 weeks of cancer progression, respectively; LT and HT denote low tumor and high tumor, respectively; PBS, phosphate-buffered saline. Different letters denote statistical significance at an alpha set a P [?] 0.05.

Figure 3. mRNA abundance of markers of extracellular matrix remodeling and turnover during the progression of cancer cachexia in females (left) and males (right). (A-B) MMP2 and MMP9 mRNA abundance in female tibialis anterior muscle. (C-D) MMP2 and MMP9 mRNA abundance in male tibialis anterior muscle. For females, *N* of 10-14 per group were used. For males, *N* of 7-8 per group were used. 1wk, 2wk, 3wk, 4wk, denotes 1, 2, 3, and 4 weeks of cancer progression, respectively; LT and HT denote low tumor and high tumor, respectively; PBS, phosphate-buffered saline. Different letters denote statistical significance at an alpha set a P [?] 0.05.

Figure 4 . mRNA abundance of the TGF-β/SMAD signaling pathway components during the progression of cancer cachexia. (A-C) Relative mRNA abundance of TGF-β1, SMAD 2/3 in female tibialis anterior muscle. (D-F) Relative mRNA abundance of TGF-β1, SMAD 2/3 in male tibialis anterior muscle. For females, *N* of 10-14 per group were used. For males, *N* of 7-8 per group were used. 1wk, 2wk, 3wk, 4wk, denotes 1, 2, 3,

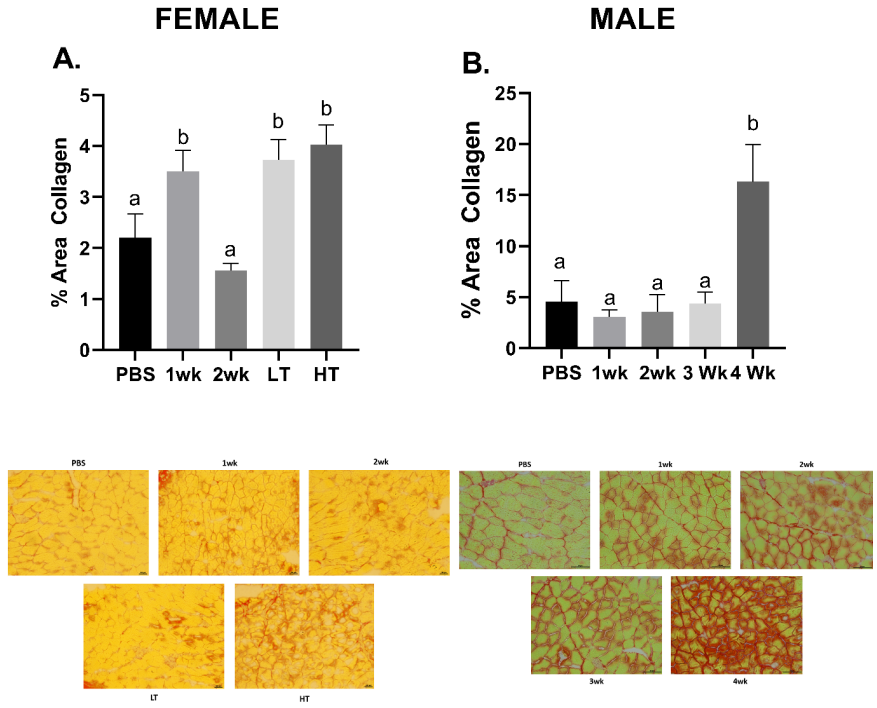
and 4 weeks of cancer progression, respectively; LT and HT denote low tumor and high tumor, respectively; PBS, phosphate-buffered saline. Different letters denote statistical significance at an alpha set a P [?] 0.05.

Figure 5. Effect of LCM treated media on C2C12 myotube size. (A) Representative images of C2C12 myotubes in media treated with vehicle or LCM. (B) Myotube diameter on C2C12 myotubes treated with either Vehicle (Veh) or LLC conditioned media (LCM). Different letters denote statistical significance at an alpha set a P [?] 0.05.

Figure 6 . Effect of LCM treated media and co-culture on ECM regulation in C2C12 myotubes. (A-C) mRNA abundance of collagen1, 3, and the collagen 3 to 1 ratio. (D-F). mRNA abundance of ECM remodeling and turnover markers MMP2, 9, and TIMP-1. Different letters denote statistical significance at an alpha set a P [?] 0.05.

Figure 7. Effect of LCM treated media and co-culture on SMAD mRNA abundance in C2C12 myotubes or 3T3 fibroblasts treated with vehicle (Veh) or LLC conditioned media (LCM) treated. Different letters denote statistical significance at an alpha set a P [?] 0.05.

Figure 1



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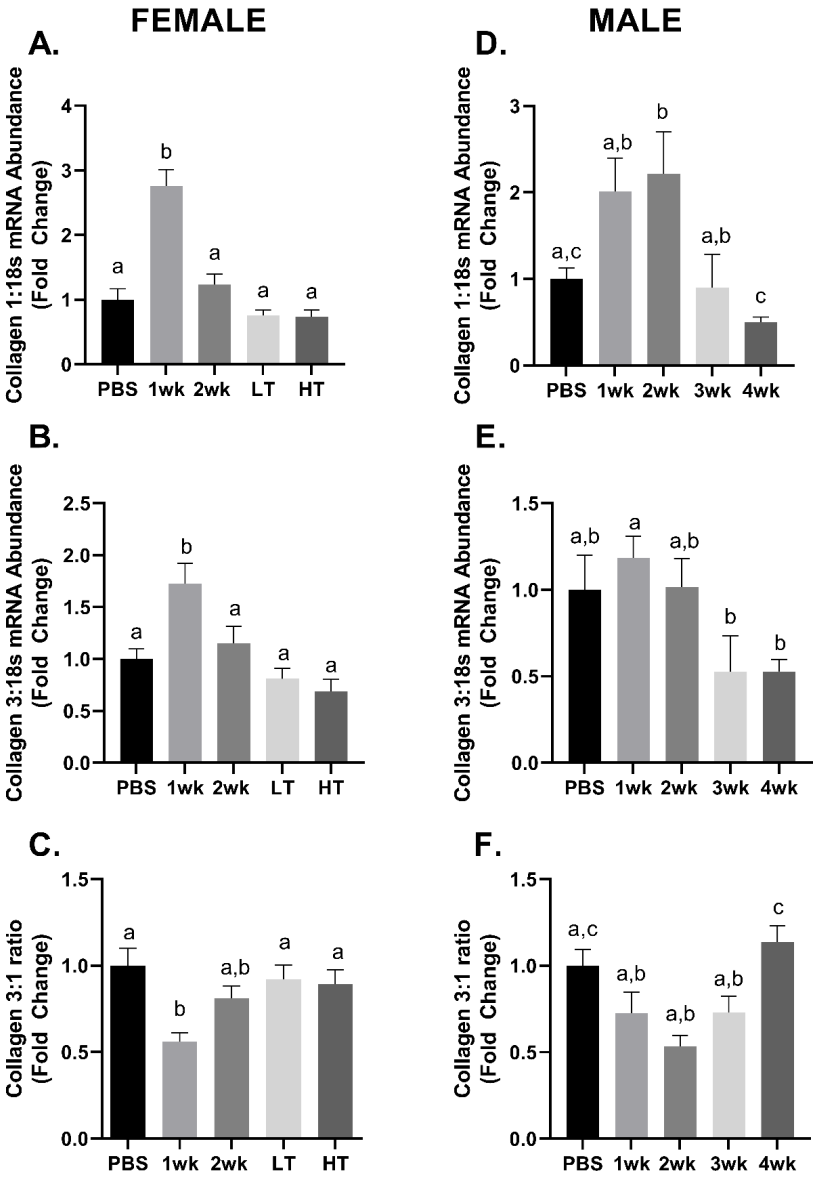

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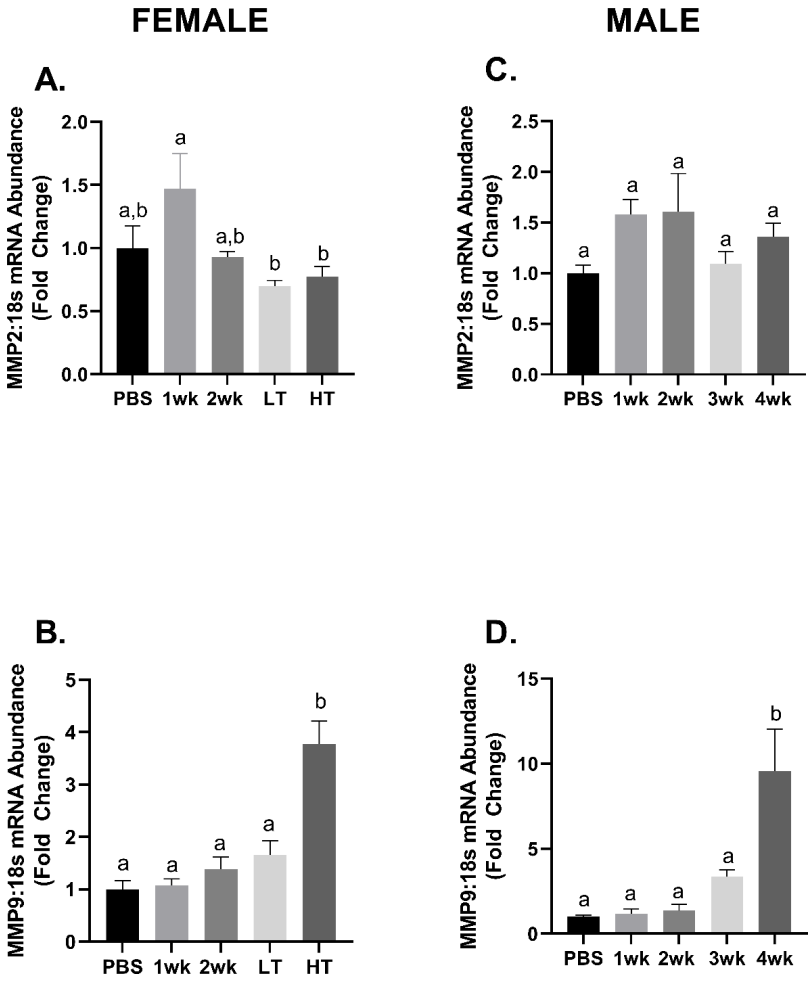

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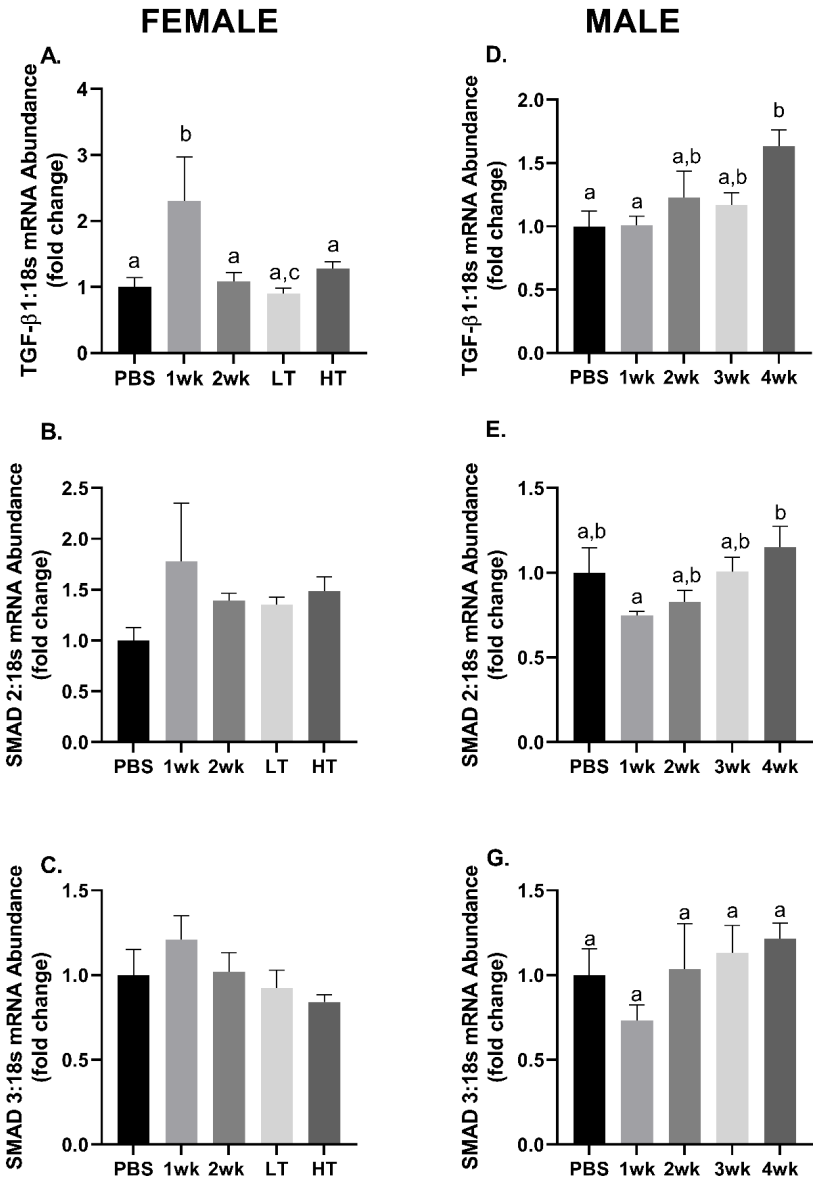


Figure 5

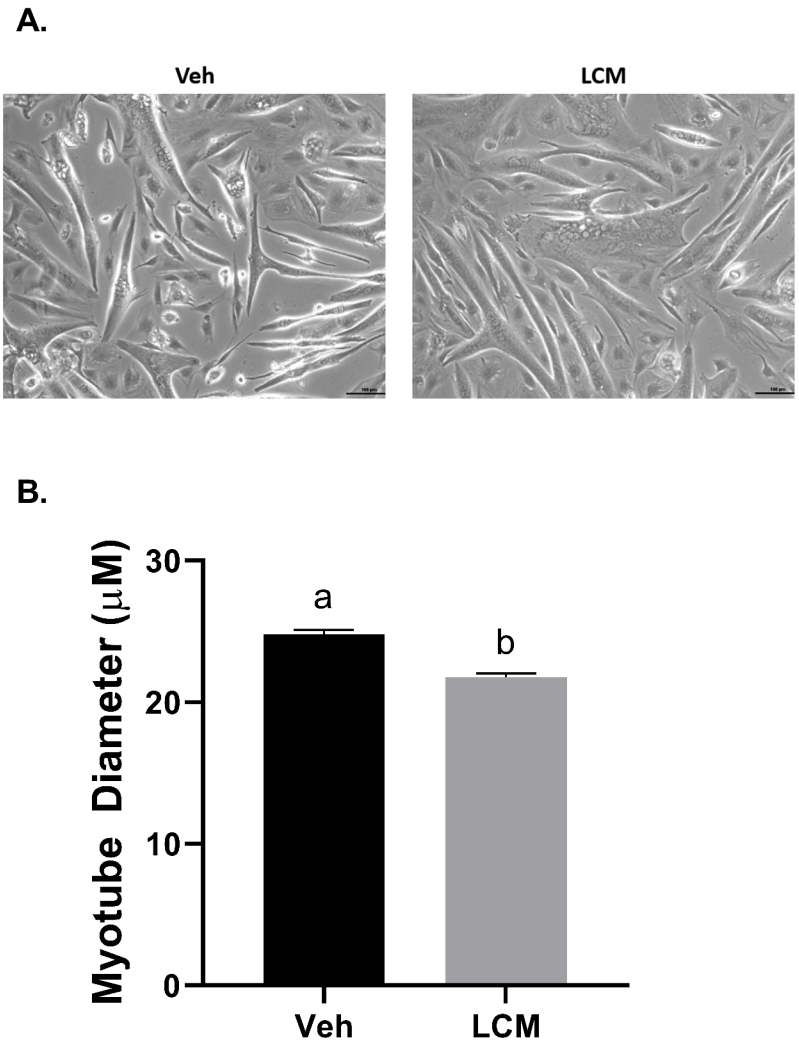


Figure 6

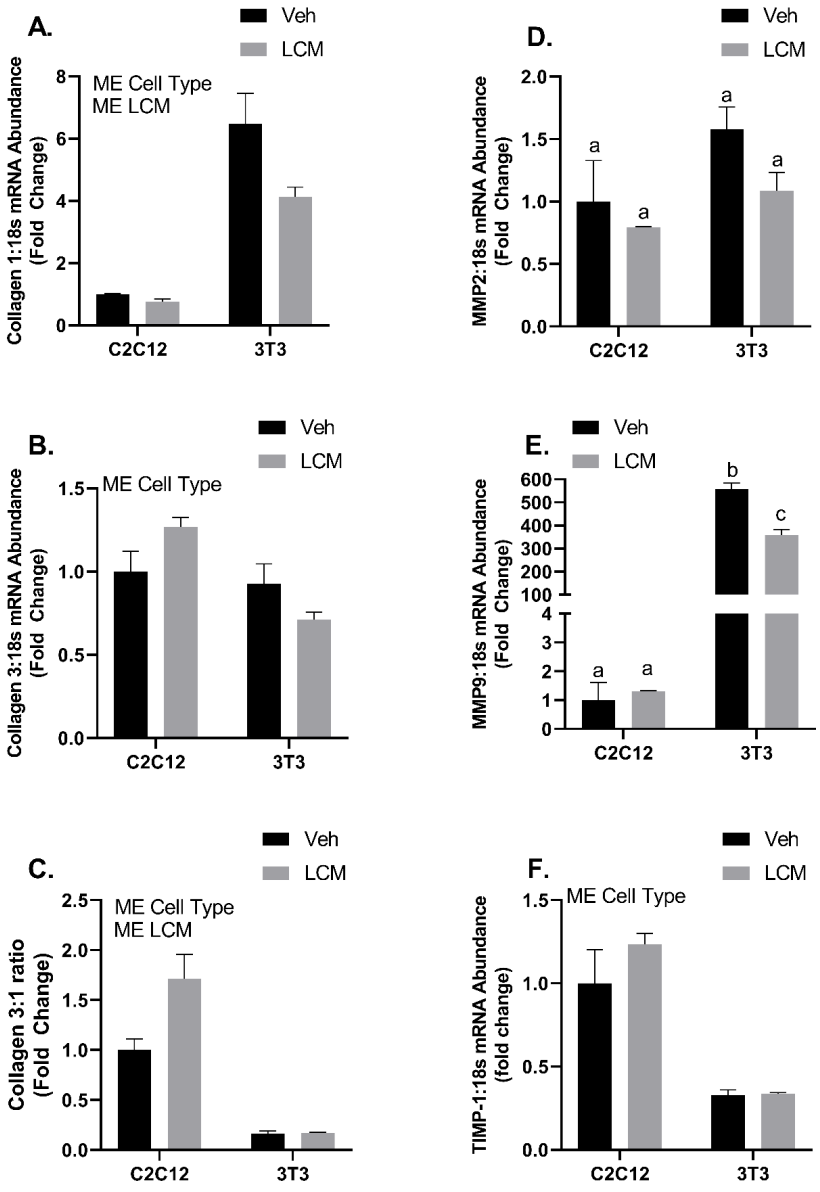
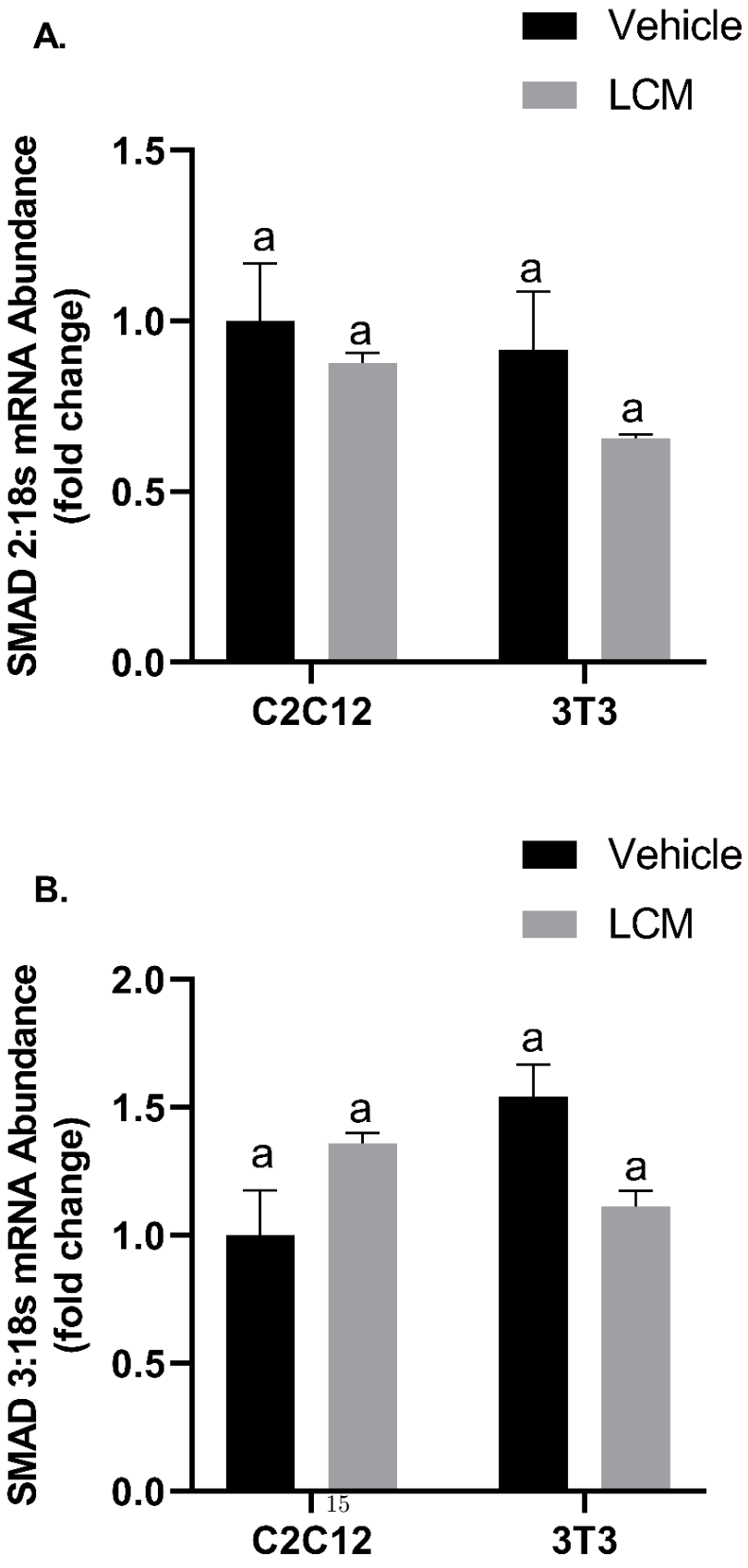


Figure 7



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