Fibro-adipogenic remodeling of the diaphragm in obesity-associated respiratory dysfunction

(Obesity-induced diaphragm remodeling)

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ABSTRACT

Respiratory dysfunction is a common complication of obesity, conferring cardiovascular morbidity, increased mortality and often necessitating mechanical ventilatory support. While impaired lung expansion in the setting of increased adipose mass and reduced central response to hypercapnia have been implicated as pathophysiological drivers, the impact of obesity on respiratory muscles—in particular, the diaphragm—has not been investigated in detail. Here, we demonstrate that chronic high-fat diet (HFD)-feeding impairs diaphragm muscle function, as assessed \textit{in vivo} by ultrasonography and \textit{ex vivo} by measurement of contractile force. During HFD time course, progressive adipose tissue expansion and collagen deposition within the diaphragm parallel contractile deficits. Moreover, intra-diaphragmatic fibro-adipogenic progenitors (FAPs) proliferate with long-term HFD-feeding while giving rise to adipocytes and type I collagen-depositing fibroblasts. Thrombospondin-1 (THBS1), a circulating adipokine, increases with obesity and induces FAP proliferation. These findings suggest a novel role for FAP-mediated fibro-adipogenic diaphragm remodeling in obesity-associated respiratory dysfunction.
INTRODUCTION

Obesity-associated respiratory complications range from simple dyspnea on exertion to life-threatening obesity hypoventilation syndrome (OHS) (1). OHS—defined by PaCO$_2$ > 45Torr in individuals with body mass index (BMI) > 30kg/m$^2$ and no alternative cause of hypercapnia—impacts 2 million Americans (2) and exacerbates risks of heart failure and early mortality (3).

OHS pathophysiology is incompletely understood; however, restricted lung expansion in the setting of excess thoracic and visceral adipose tissue is assumed a major driver (4). Clinical studies demonstrate respiratory muscle weakness parallels this physical restriction (5-6), while autopsy samples from OHS patients contain prominent intra-diaphragmatic adipocyte inclusions (7). These findings suggest anatomic remodeling of the diaphragm itself may contribute to obesity-induced respiratory impairment.

Pre-clinical studies of respiratory function in obesity have largely employed genetically obese Zucker diabetic fatty (ZDF) rats. In this model, ex vivo diaphragmatic testing has yielded equivocal results, with contractile reduced force in older animals (8), but unchanged (9-10) or increased (11) in younger animals. Ob/ob mice demonstrate hypoventilation reversible by leptin infusion, indicating this adipokine may regulate central respiratory drive (12). Nonetheless, ventilatory function in diet-induced obese (DIO) models is unaffected by this maneuver (12), suggesting exploration of other mechanisms, including intrinsic diaphragm compromise, is warranted to better understand the pathogenesis of obesity-induced respiratory dysfunction.

Accumulation of intramuscular adipose tissue (IMAT) is a complication of immobility (13) and muscular dystrophy (14). IMAT increases with normal aging (15) and quantitatively correlates with reduced muscle strength in the elderly (16). Recent studies demonstrate IMAT expansion is also associated with weakness in obese and type 2 diabetic individuals (17-19).
Intramuscular fibrosis accompanies impaired regeneration, increased tissue stiffness and reduced contractile force in skeletal muscle disorders (20). Intramuscular extracellular matrix (ECM) deposition is associated with insulin resistance in obese mice (21-23), while up-regulation of skeletal muscle collagen gene expression occurs in humans after lipid infusion (24), experimental overfeeding (25), and with chronic obesity (26). Despite links between over-nutrition and ECM remodeling, direct effects of these fibrotic changes on muscle contraction remain largely undefined. Thrombospondin-1 (THBS1), a circulating ECM protein, activates transforming growth factor beta (TGFβ) (27), promotes mesenchymal cell proliferation (28) and underlies fibrosis in limb muscles of obese mice (21).

Fibro-adipogenic progenitors (FAPs) are mesenchymal cells residing within skeletal muscle that give rise to adipocytes and fibroblasts in mice and humans (29-31). Largely quiescent at baseline, FAPs proliferate in response to muscle injury, facilitating muscle regeneration (32-33). In the mdx mouse model of muscular dystrophy, disordered FAP dynamics contribute to pathological adiposity and fibrosis (34-35). While FAPs may remodel skeletal muscle in obesity, their response to metabolic challenge remains unexamined. In this study, we tested whether fibro-adipogenic diaphragm remodeling occurs in obesity-associated respiratory impairment and whether FAPs contribute to the process.

METHODS

Animals

Mice were obtained from Jackson Laboratory (Bar Harbor, ME). Jackson maintains Sca-1 GFP (#12643), PDGFRα-Cre (#013148) and ob/ob (#000632) mice on a C57BL/6J, and mT/mG (#007576) mice on a 129X1/SvJ background. Animals were maintained in pathogen-free housing with 12-hour light-dark cycle and ad libitum food/water. For DIO studies, mice received
normal chow diet (CD) (5L0D; LabDiet, St Louis, MO) until 8-weeks-old. Control mice continued CD, while experimental mice switched to high-fat diet (HFD) containing 45% calories from lipid (D12451; Research Diets, New Brunswick, NJ). Mice consumed CD or HFD for 1, 3, or 6 months before analyses. For comparisons of \( \text{ob/ob} \) and wild type (WT) \( C57BL/6J \) mice, all animals consumed CD and were analyzed at 16-weeks-old. The University of Michigan Institutional Animal Care and Use Committee approved all studies.

**Diaphragm ultrasonography**

As described in (36), diaphragms were localized by ultrasound (US) using a transversely-oriented MS250 transducer (frequency 24MHz) (Vevo 2100; Visual Sonics, Toronto, ON). Diaphragm motion, observed in M-mode, was recorded \( \geq 3 \) respiratory cycles. Excursion amplitude, inspiratory duration, inspiratory velocity, expiratory velocity and peak-to-peak time were measured on still images. Inspiratory duty cycle was calculated as quotient of inspiratory duration/peak-to-peak time.

**Ex vivo isometric testing**

Twitch properties and tetanic force (37) were measured on 2-4mm-wide lateral costal diaphragm strips. In a Krebs Ringer bath containing 0.03mM tubocurarine chloride, held at 25°C and bubbled with 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \) (maintaining pH 7.4), an attached rib was sutured to a servomotor (model 305B; Aurora Scientific, Aurora, ON) and the free central tendon edge to a force transducer (model BG-50; Kulite Semiconductor Products, Leonia, NJ). The bath was electrically stimulated through a field generated between 2 platinum electrodes by a biphasic current stimulator (model 701A; Aurora Scientific). LabVIEW 2014 software (National
Instruments, Austin, TX) controlled electrical pulse properties and servomotor activity while recording transducer data. Strips were adjusted to optimal length ($L_o$) at which a stimulus pulse elicited maximum force. Resting tension was measured at $L_o$. Contraction and half-relaxation times were measured during individual isometric twitches. Muscle cross sectional area (CSA) was calculated using $L_o$ and muscle mass. Specific force was calculated as quotient of $P_o$/CSA.

**Stromal cell analysis**

Diaphragmatic stromal cells were isolated through a protocol adapted from (30). Costal diaphragms were excised, minced and digested in collagenase type 2 (Worthington Biochemical, Lakewood, NJ) diluted to 0.067% by weight in serum-free DMEM (Thermo Fisher, Waltham, MA). After 1-hour at 37°C, collagenase was inactivated with DMEM containing 10% fetal bovine serum (FBS). Resultant suspensions were sequentially passed through 100µm and 40µm cell strainers.

Flow cytometry used established marker profiles (29). Briefly, cells isolated from $Sca-1$ *GFP* mice were fixed with 4% PFA, permeabilized/blocke in 0.3% Triton X-100/5% normal goat serum, then incubated with fluorophore-conjugated CD31, CD45, integrin $\alpha_7$ and PDGFR$\alpha$ antibodies ([Table S1](#)). After DAPI staining, cells were analyzed on a MoFlo Astrios EQ running Summit software (Version 6.3; Beckman-Coulter, Brea, CA). Gates were established using fluorescence minus one for labeled antibodies and WT littermate for ($Sca-1$) GFP. Cell cycle analysis was performed based on nuclear DAPI staining. For gene expression and *ex vivo* analyses of stromal isolates from WT mice, cells were purified by 2-step magnetic-assisted cell sorting employing MiniMACS® columns (Miltenyi, Bergisch Gladbach, GER) following manufacturer’s instructions. Cells were first subjected to negative selection using Lin (CD31,
CD45) antibodies (130-09-858). Resulting Lin$^-$ cells were sequentially incubated with FITC-conjugated Sca-1 antibody (120-003-751) and anti-FITC microbeads (120-003-754). Lin$^-$/Sca-1$^+$ (FAP), and Lin$^-$/Sca-1$^-$ (non-FAP)-enriched populations were subsequently analyzed.

**Gene expression analysis:**

Whole tissues—costal diaphragm and brown adipose tissue (BAT)—were cleaned of adherent tissues, snap frozen and digested in Trizol (Thermo Fisher, Waltham, MA) with mechanical homogenization; while stromal cells subjected to MACS isolation were pelleted by centrifugation. Both underwent RNEasy-based RNA isolation following manufacturer’s instructions (Qiagen, Germantown, MD). cDNA synthesis with SuperScript II (Invitrogen, Carlsbad, CA) preceded quantitative PCR with SYBR Green (Thermo Fisher, Waltham, MA) or Taqman (Applied Biosystems, Foster City, CA) on a StepOnePlus machine (Applied Biosystems, Foster City, CA). Primer/probe sequences are indicated in Table S2.

**Cell culture:**

After isolation, stromal cells were cultured for 1 day in 24-well plates containing standard medium—DMEM with 10% FBS and antibiotic/antimycotic (penicillin, streptomycin, amphotericin B; Sigma, St Louis, MO). Cells were then seeded at 30% confluence in 4-well chamber slides (Lab Tek, Thermo Fisher, Waltham, MA) with standard medium. Those allocated for Ki67 and extracellular collagen staining were evaluated after 3 days. Those allocated for adipogenesis were cultured for 5 days (reaching full confluence), then switched to standard medium plus adipogenic cocktail (1µg/mL insulin, 250nM dexamethasone, 10µM troglitazone and 10nM triiodothyronine) for 7 days, then to standard medium plus insulin (1µg/mL) for 3
days. After each experiment, cells were fixed, blocked and immunostained as previously described (38). For Ki67 staining, wash buffers and antibody diluents contained 0.2% Tween-20. This was excluded for extracellular collagen staining. Cells subjected to adipogenesis analysis were fixed then incubated in BODIPY-543 (Thermo Fisher, Waltham, MA). Cells subjected to THBS1 treatment were transferred to slide wells containing standard medium for 12 hours, then switched to DMEM with 1% FBS. Subsequently, individual groups were incubated in this medium +/-THBS-1 (Calbiochem, San Diego, CA; #605225) at 0, 1 or 5\( \mu \)g/mL; followed by Ki67 staining. Counter-stain with Alexa Fluor 488-conjugated phalloidin (Thermo Fisher) was used in specific experiments.

**Decellularization/ oil red O staining:**

As previously described (39), whole diaphragms were decellularized in 1% sodium dodecyl sulfate (SDS) for 5 days, fixed in 4% paraformaldehyde (PFA), then incubated in oil red O dissolved to 0.3% by weight in 60% isopropanol. After overnight incubation in 1% SDS, they were affixed to wax plates or dissection petri dishes for analysis.

**Histological analysis:**

Six-\( \mu \)m-thick formalin-fixed, paraffin-embedded (FFPE) sections of costal hemidiaphragm, BAT, inguinal and gonadal white adipose tissue (WAT) were prepared as previously described (21) and stained with sirius red, hematoxylin & eosin (H/E) and Masson’s trichrome by standard procedures. Immunohistochemistry on FFPE sections was performed as described in (21), with primary and secondary antibodies indicated in (Table S1). Full-thickness diaphragm chunks (4 mm\(^3\)) fixed in 1% PFA for 5 minutes at room temperature were used for
immunofluorescent detection of type I and VI collagen by whole-mount confocal microscopy. All processing was performed in detergent-free solutions. Samples were imaged using an Olympus DP72 camera mounted on an Olympus SZ61 microscope (Tokyo, JP) or a Nikon A1 confocal microscope running NIS-Elements software (Olympus). Three non-consecutive sections per animal were used for quantitative morphometry, with analysis performed using NIH ImageJ.

**Western blot:**

Plasma (1µL) was diluted in SDS lysis buffer, denatured, and run in 5% SDS-PAGE. THBS1 was detected by Western blot using rabbit anti-THBS1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (21).

**Statistical analysis**

Data analysis employed Student’s t-test for 2-group comparisons, 1-way ANOVA (with Tukey post hoc test) for >2-group comparisons, 2-way ANOVA (with Sidak post hoc test) for 2 variables, and linear regression for correlation analysis. p value <0.05 indicated statistical significance. Data are shown as mean +/- standard deviation.

**RESULTS**

**Diaphragm motion compromise with long-term HFD challenge**

To generate a model of obesity-induced respiratory dysfunction, we placed male C57BL/6J mice on a 6-month DIO time course, observing marked weight gain (Fig 1A) and glucose intolerance (Fig S1A) in HFD-fed animals versus CD-fed counterparts. We analyzed diaphragm motion with non-invasive US (Fig 1B). This technique is validated in rodents, and US-based measurement of diaphragm excursion amplitude correlates with *ex vivo* isometric force.
values in WT and *mdx* mice (36). In our animals, amplitude was unchanged between groups at 3 months, but significantly lower in HFD-fed animals after 6 months (Fig 1C). Similar HFD-induced declines occurred in inspiratory and expiratory velocity (Fig 1D-E). Additionally, 6-month HFD mice displayed increased respiratory rates, indicated by reduced peak-to-peak time (Fig 1F). Duty cycle, denoting percentage of each breath spent in inspiration (inspiratory duration/peak-to-peak time), also increased significantly with 6-month HFD feeding (Fig 1G). These data indicate that long-term DIO impairs diaphragm motion, inducing restrained, low-amplitude contractions occurring at shorter intervals.

We analyzed diaphragm motion in *ob/ob* mice, which at 16-weeks-old, had weights comparable to those of 6-month DIO animals (Fig S2A). When compared to age-matched WT controls, *ob/ob* mice also showed reduced amplitude (Fig S2B), inspiratory velocity (Fig S2C), expiratory velocity (Fig S2D) and peak-to-peak time (Fig S2E); however, duty cycle was unchanged (Fig S2F). As such, *ob/ob* animals displayed similar, but not identical, diaphragm motion defects versus DIO mice.

**Intrinsic diaphragm contractile compromise in long-term DIO**

We next tested whether observed ultrasonographic changes resulted from functional defects intrinsic to the diaphragm. We specifically evaluated its costal region (40) (Fig S1B)—previously shown responsible for contractile force generation (41)—by performing *ex vivo* isometric testing (Fig S1C). Indeed, specific force, defined as maximum tetanic force per unit CSA, was more than 15% lower in 6-month HFD- versus CD-fed mice (Fig 1H). Specific force measurements from individual animals correlated with excursion amplitudes (Fig S1D) and exhibited negative linear relationships with body weight (Fig S1E) and inguinal adipose mass
(Fig S1F). Resting tension, time to peak tetanus and half-relaxation time were unchanged with HFD-feeding (Fig S1G-I). Notably, the reduced force generation induced by HFD-feeding was not associated with significant changes in costal diaphragm thickness, average myofiber diameter, or percentage of muscle cross-sectional area (CSA) occupied by myofibers (Fig S1J-M). In contrast with DIO mice, specific force measurements from ob/ob diaphragms were nearly identical to those of controls (Fig S2G). This result parallels prior reports of normal diaphragm contractile force in young ZDF rats (9-10) and is consistent with findings that ob/ob hypoventilation can be quickly reversed by leptin infusion (12).

**DIO induces progressive intra-diaphragmatic adiposity**

Since long-term DIO yielded intrinsic diaphragmatic contractile compromise without obvious differences in myofiber morphology, we assessed whether other anatomic changes accompanied physiologic impairment. First, we analyzed adiposity using decellularized diaphragms of 6-month CD and HFD-fed mice. In CD-fed animals, oil red O (ORO) staining revealed well-demarcated lipid droplets closely juxtaposed with apparent branches of the phrenic vasculature (40) (Fig 2A-B). HFD-feeding increased ORO-positive droplet number and size, especially in the lateral costal diaphragm (Fig 2A-B). H&E staining of costal diaphragm sections also showed unilocular adipocytes (Fig 2C), their identity confirmed by perilipin immunohistochemistry (Fig 2D). These localized near blood vessels in CD-fed mice but extended farther into the tissue with HFD-feeding (Fig 2C). CSA of individual adipocytes increased after 1-month HFD-feeding (Fig 2E) and was 3-fold greater versus controls at 3 and 6 months (Fig 2E). Size distribution profiling demonstrated adipocytes in CD-fed diaphragms to be small, the majority with CSAs≤250µm² (Fig 2F). On the contrary, HFD diaphragms contained
many with CSA≥1000µm² (Fig 2F). Adipocyte number was statistically unchanged between groups at 1 and 3 months; however, significantly elevated with HFD-feeding at 6 months (Fig 2G). Percentage of diaphragm CSA occupied by adipocytes also increased significantly with HFD-feeding only at the 6-month time point (Fig 2H). Notably, intra-diaphragmatic adipocyte number negatively correlated with specific force in individually-assessed diaphragm strips (Fig 2I).

Transcripts encoding mature adipocyte markers fatty acid binding protein (FABP4) and leptin, as well as a WAT marker TCF21 (42), increased with HFD-feeding. On the contrary, those encoding lipoprotein lipase (LPL), adiponectin, and PPARγ were unchanged. As seen in other adipose depots (43), diaphragmatic adipsin declined with DIO (Fig 2J). WAT exhibits obesity-induced inflammation and macrophage infiltration (44). While DIO diaphragms showed no change in Il1b, Tnfa and Il6 (Fig S3A); Emr1, encoding macrophage marker F4/80, increased marginally with HFD-feeding (Fig S3A). Nonetheless, we observed few F4/80⁺ cells and no discernable crown-like structures in diaphragms of CD or HFD-fed mice (Fig S3B).

Interestingly, DIO diaphragms showed strong Ucp1 induction, albeit at a low level versus BAT (Fig 1K). Consistent with this, immunohistochemistry revealed isolated UCP-1⁺ unilocular adipocytes among many UCP1⁻ cells (Fig 1L). Together, these findings suggest intra-diaphragmatic IMAT exhibits WAT-like characteristics and ability to express UCP-1 after metabolic challenge (45).

Notably, Diaphragms of ob/ob mice, which demonstrated no ex vivo contractile defects, had larger adipocytes (Fig S2H-I) but equivalent adipocyte number relative to WT controls (Fig S2J).
Progressive diaphragm fibrosis in DIO

Given association of intramuscular ECM deposition with obesity (21-26) and contractile dysfunction (20), we postulated DIO diaphragms might undergo fibrotic remodeling. Indeed, percentage of costal diaphragm CSA occupied by polymerized collagen progressively increased during HFD-feeding (Fig 3A), becoming statistically elevated versus controls by 6 months (Fig 3B). With DIO, collagen deposition was especially apparent near adipocytes (Fig 3C). Moreover, within strips subjected to isometric testing, polymerized collagen area negatively correlated with specific force measurements (Fig 3D).

Gene expression analysis of 6-month costal diaphragms revealed HFD-induced increases in transcripts encoding type I and VI collagen (Fig 3E). On the contrary, levels of those encoding type III and IV collagen, as well as collagen crosslinking enzyme lysyl oxidase (LOX), were unchanged between groups (Fig 3E-F). Among matrix metalloproteinases (MMPs), Mmp2 declined, Mmp9 did not change, and Mmp14 increased with DIO (Fig 3G). Expression of profibrotic mediator Tgfb1 was unchanged, while that of Tgfb3 increased in HFD-fed mice (Fig 3H). Additionally, levels of Pdgfa and Fgf2 were unchanged, while those of Ctgf and Thbs1 increased with HFD-feeding (Fig 3I). These profiles suggest active and complex gene regulation underlying diaphragm ECM remodeling in long-term DIO.

Unlike DIO animals, ob/ob mice showed no increase in intra-diaphragmatic collagen deposition versus WT controls (Fig S4).

Fibro-adipogenic progenitors proliferate in association with intra-diaphragmatic adiposity and fibrosis
We surmised that FAPs—skeletal muscle progenitors capable of differentiation into adipocytes and fibroblasts (29-30)—might underlie the fibro-adipogenic diaphragmatic remodeling observed in DIO mice. FAPs express PDGFRα and can be defined by a surface marker profile positive for Sca-1 and negative for CD31 (endothelial marker), CD45 (hematopoietic marker), and integrin α7 (satellite cell marker) (29-30). We intercrossed PDGFRα-Cre mice, which express Cre recombinase under the PDGFRα promoter, with mT/mG reporter mice to trace distribution of GFP+ (FAP-derived) cells within the costal diaphragm (Fig 4A). Significantly, GFP+ cells constituted all adipocytes in 6-month CD and HFD-fed PDGFRα Cre-mT/mG samples (Fig 4B, S5A). These adipocytes were larger and more abundant with HFD-feeding (Fig 4B). We then assessed distribution of extracellular type I collagen (COL1) and type VI collagen (COL6) within costal diaphragms of the same animals. We noted expected collagen staining in epimysium and central tendon, while observing no signal in samples stained with control antibody (Fig S5 B-D). CD costal diaphragms contained thin COL1 fibrils between myofiber bundles (Fig 4C). COL1 bands appeared larger in HFD-fed samples, while irregular densities also surrounded clusters of GFP+ stromal cells and adipocytes (Fig 4C). Bands of COL6 paralleled myofibers in both groups; however, HFD-fed diaphragms also showed prominent signal surrounding adipocyte inclusions (Fig 4C).

Since PDGFRα+ cells gave rise to all adipocytes and many collagen-depositing cells, we specifically analyzed response of intra-diaphragmatic FAPs to HFD-feeding at the 6-month time point. First, we noted increased costal diaphragm PDGFRα expression in HFD-fed mice (Fig 4D). Next, we isolated stromal cells from costal diaphragms of Sca-1 GFP mice. These animals harbor an EGFP construct driven by Sca-1 promoter, enabling analysis of Sca-1+/CD31−/CD45−/integrin α7− FAPs by flow cytometry (Fig S6A-B). Consistent with prior
reports, we found these cells were broadly PDGFRα⁺ (Fig S6A). In DAPI-stained stromal cell isolates, we found both FAP number and proliferation dramatically increased with long-term obesity (Fig 4E).

**Obesity-induced FAP proliferation and collagen deposition**

To explore the link between DIO-induced diaphragm remodeling and FAP dynamics, we analyzed gene expression profile and *ex vivo* fibro-adipogenic potential of these cells. Specifically, we used immunomagnetic beads to isolate stromal populations enriched for FAPs (Lin⁻/Scaf1⁺) and non-FAP stromal cells (Lin⁻/Scaf1⁻) from costal diaphragms of WT 6-month CD and HFD-fed mice. Lin⁻/Sca1⁺ FAPs preferentially expressed *Pdgfra*, while Lin⁻/Sca1⁻ non-FAPs preferentially expressed satellite cell marker *Pax7* (Fig 5A). Critically, HFD-feeding induced elevated expression of *Col1, Col3* and *Col6* in FAPs but not non-FAPs, implicating the former as the likely source of increased tissue-level collagens (Fig 5A). Interestingly, *Tgfβ3*, also globally elevated in the DIO costal diaphragm (Fig 3H), increased with HFD-feeding in both populations (Fig 5A), suggesting FAP-mediated fibrosis may be regulated by multiple stromal cell types.

Consistent with *in vivo* data, we observed greater *ex vivo* proliferation (Ki67 staining) in FAPs isolated from HFD-fed mice (Fig 5B). COL1 deposition also increased markedly in these cells (Fig 5C). Analysis of unsorted stromal cells from *PDGFRa Cre-mT/mG* mice confirmed these findings, with increased GFP⁺ cell proliferation and collagen deposition seen in DIO costal diaphragm isolates (Fig S7).

In FAPs, HFD-feeding induced expression of *Tcf21, Il6*, and *Ucp1*, but not classic early adipogenesis-related genes *Pparg2, Cebpa*, and *Pgc1a* (Fig 5D). In agreement with this, FAPs
from CD- and HFD-fed mice showed equivalent *ex vivo* adipogenesis (Fig 5E). Along with our *in vivo* findings, these data indicate that DIO induces FAP proliferation and collagen deposition but does not impact adipogenic potential. Increased diaphragmatic adiposity after long-term HFD-feeding therefore likely results from increased FAP number.

We next sought trophic factors that might underlie FAP proliferation in the obese diaphragm. We focused on THBS1, a circulating ECM protein, produced by visceral adipose tissue (21) that acts as a potent mesenchymal cell mitogen (28). Its genetic ablation protects DIO mice from skeletal muscle fibrosis and insulin resistance (21). We first analyzed serum THBS1 level, finding it markedly increased in 6-month HFD-fed mice (Fig 5F). We next treated isolated FAPS with increasing THBS1 concentrations over a physiological range seen in obese humans (46). FAPs from CD fed mice exhibited dose-dependent proliferation, whereas those from HFD-fed mice showed enhanced proliferation even at the 1 µg/ml concentration. These data highlight THBS1 as a candidate regulator of FAP proliferation and fibro-adipogenic remodeling of the DIO diaphragm.

**DISCUSSION**

In this study, we demonstrated that the mouse diaphragm undergoes progressive fibro-adipogenic remodeling with long-term HFD-feeding, ultimately exhibiting impaired motion *in vivo* and contraction *ex vivo*. The temporal correlation between fibro-adipogenic changes and diaphragm dysfunction, coupled with identification of proliferating FAPs as a likely remodeling driver, provides a framework for better understanding respiratory impairment in obesity.

Our data demonstrate DIO of considerable duration is needed to cause respiratory compromise in mice. Non-invasive US found no significant diaphragm motion changes after 3
months HFD-feeding; but revealed shallow, short-interval breaths with increased duty cycle by 6 months. This approximates breathing patterns of obese humans (1, 5). As previously shown in mdx mice (a muscular dystrophy model with primary diaphragm dysfunction) (36), low excursion amplitudes seen on US correlated with reduced specific force measured ex vivo. In turn, specific force correlated negatively with body weight and adiposity measures. This parallels the increasing risk of obesity-associated respiratory dysfunction clinically observed at higher BMI (2). Elevated intra-diaphragmatic adipocyte number and polymerized collagen content emerged only at the 6-month time point, when diaphragm dysfunction manifested, and correlated quantitatively with specific force decrements.

Equally obese but relatively young (16-week-old) leptin-deficient ob/ob mice showed similar diaphragm motion defects, with lower amplitude, inspiratory velocity and expiratory velocity; however, had nearly identical duty cycle and ex vivo specific force versus age-matched controls. Ob/ob diaphragms also did not demonstrate increased adipocyte number and polymerized collagen seen with long-term DIO. These findings suggest hypoventilation in ob/ob mice occurs independent of primary diaphragm compromise. Indeed, leptin is a key regulator of central respiratory control (47) and its administration can reverse ventilatory defect in ob/ob but not DIO mice (12). We therefore surmise chronicity of HFD-feeding, with potential contribution from dietary content, underlies diaphragm anatomic remodeling and contractile dysfunction not seen in the younger ob/ob mice. The premise that long-term obesity is needed to induce intrinsic diaphragm defects may explain discrepancies in previous analyses of ZDF rats. In that model, while diaphragm isolates from young animals (3-4-months-old) demonstrate unchanged or even increased (9-11) isometric force, those from older animals (8-months-old) show force reduction (8).
IMAT accumulation and tissue fibrosis are both associated with skeletal muscle contractile dysfunction (16-20) and occur in obesity (17-19, 21-26). In our DIO model, FAPs emerged as likely drivers of diaphragm fibro-adipogenic remodeling, giving rise to all adipocytes and many collagen-producing fibroblast-like cells. HFD-feeding caused FAPs to proliferate and assume a fibrogenic phenotype. Since FAPs from HFD-fed mice had similar adipogenic potential versus controls, we reason progressive adipocyte accumulation in long-term DIO results from slow but progressive adipocyte differentiation from within a larger FAP pool. FAPs from HFD-fed mice were nonetheless qualitatively different, exhibiting elevated expression of specific adipocyte-related genes. Tcf21, a WAT marker (42), recently shown enriched in visceral adipose stem cells (38), was induced in FAPs with HFD feeding; as was Il6, a TCF-21-regulated gene (38). HFD-feeding also upregulated FAP-specific expression of Ucp1, corresponding with presence of scattered UCP-1+ unilocular adipocytes in DIO diaphragms. Interestingly, Ucp1 upregulation in FAPs and UCP-1+ adipocytes were identified in mouse tibialis anterior muscles subjected toxin-induced injury (48). Specific contributions of increased intramuscular adiposity and fibrosis to contractile dysfunction remain elusive; however, represent tractable areas of inquiry with improved understanding of FAP dynamics in obesity.

Our identification of THBS1—a WAT-derived, circulating ECM protein increased in obesity and type 2 diabetes (46)—as a mediator of FAP proliferation provides one link between over-nutrition and diaphragm fibro-adipogenic remodeling. Recent work has identified THBS1 as a stromal cell mitogen, acting in concert with known FAP growth factors TGFβ and PDGF-AA (28). Since THBS1 also regulates skeletal muscle fibrosis (21), further evaluating its impact on FAPs will enable novel mechanistic exploration of diaphragm structure/function compromise in obesity.
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Duality of Interest
No potential conflicts of interest relevant to this article were reported.

Author contributions
EDB and THC conceived of the research idea and designed experiments. EDB, KCB, CSD and TA performed the experiments. TA, FH, SVB and DEM provided technical consultation. EDB, SVB, DEM and THC evaluated the data. EDB and THC wrote the manuscript and all authors reviewed the manuscript.

Guarantor statement
THC is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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FIGURE LEGENDS

Figure 1. Long-term DIO impairs diaphragm function. (A) Total body weight (g) of control diet (CD) and high fat diet (HFD)-fed mice after 1 month (1m), 3 months (3m) and 6 months (6m) feeding. (B) M-mode ultrasound (US) tracing of diaphragmatic motion with measured parameters labeled. (C) Amplitude (mm). (D) Inspiratory velocity (mm/s). (E) Expiratory velocity (mm/s). (F) Peak to peak time (ms). (G) Duty cycle (inspiratory duration/peak to peak) (percent). (H) Specific force (kN/m²) measured on 2-4 mm-wide costal diaphragm strips isolated from CD and HFD-fed mice at the 6m time point. Each point represents mean value of measurements taken from 2 strips per animal. n = 7 CD, 4 HFD mice at 1m time point; 8 CD, 6 HFD-fed mice at 3m time point; 5-9 CD, 6 HFD-fed mice at 6m time point. Data analysis employed Student’s t-test for 2-group, single variable comparisons; and 2-way ANOVA (with Sidak’s post hoc test) for 2-variable comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Figure 2. DIO causes progressive intra-diaphragmatic adiposity. (A) Decellularized whole diaphragms from 6-month (6m) control diet (CD) and high fat diet (HFD)-fed mice stained with oil red O. Representative images from 4 animals per group. (B) Decellularized diaphragm (from same groups) demonstrating lateral costal region under trans-illumination. Filled arrowheads (in CD sample) indicate adipocyte distribution near putative phrenic vasculature. Open arrowheads (in HFD) show a conglomeration of large lipid droplets. Scale 400 µm. (C) H/E-stained sagittal sections of CD and HFD costal diaphragms at 1-month (1m), 3-month (3m) and 6-month (6m) time points. Closed arrowheads (in CD sample) indicate blood vessels (frequently seen in close opposition to adipocytes). Representative images from 4-6 animals per group. Scale 25 µm.
Perilipin stained sagittal costal diaphragm sections from 6m CD and HFD-fed mice. Representative images from 4 animals per group. Scale 25 µm. (E) Average cross-sectional surface area of individual costal diaphragm adipocytes (µm ) in CD and HFD-fed mice at 1m, 3m, 6m time points. n = 4 mice per group at each time point. (F) Costal diaphragm individual adipocyte cross-sectional surface area shown as frequency distribution for 1m, 3m and 6m CD and HFD-fed mice. n = 4 mice per group at each time point. (G) Number of costal diaphragm adipocytes per mm muscle cross-sectional tissue surface area at 1m, 3m and 6m time points. n = 4 mice per group at each time point. (H) Percentage costal diaphragm cross-sectional surface area occupied by adipocytes at 1m, 3m and 6m time points. n = 4 mice per group at each time point. (I) Correlation plot showing relationship between adipocyte number (per mm cross-sectional surface area) and measured specific force in costal diaphragm strips subjected to isometric force testing. n = 6 CD and 7 HFD strips from animals at 6m time point. (J) Relative mRNA expression of transcripts encoding leptin (Lep), fatty acid binding protein (FABP)-4 (Ap2), lipoprotein lipase (Lpl), adiponectin (Adipoq), PPARγ-2 (Pparg2), adipsin (Adipsin) and TCF-21 (Tcf21) in whole costal diaphragms of CD and HFD-fed mice at the 6m time point. n = 9 mice per group. (K) mRNA level of transcript encoding uncoupling protein (UCP)-1 (Ucp1) in costal diaphragm of CD and HFD-fed mice from the 6m time point; and in brown adipose tissue (BAT) from 6m HFD-fed mice. n = 9 CD and HFD-fed diaphragm samples, 5 BAT samples. (L) Immunohistochemistry for UCP-1 in 6m CD and HFD costal diaphragm and 6m HFD-fed BAT and inguinal adipose tissue (IAT). Representative images from 4 animals per group. Scale 50 µm.

Data analysis employed Student’s t-test for 2-group, single variable comparisons; 2-way ANOVA (with Sidak’s post hoc test) for 2-variable comparisons; and linear regression for correlation testing. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 3. Progressive diaphragmatic fibrosis with DIO. (A) Sirius red-stained costal diaphragm sections from 1-month (1m), 3-month (3m) and 6-month (6m) control diet (CD) and high fat diet (HFD)-fed mice viewed under bright field and polarized light. Closed arrowheads indicate epimysial collagen, open arrowheads indicate perivascular collagen—both excluded from quantitative analysis. Representative images from 4 animals per group at each time point. Scale 50 µm. (B) Quantification of sirius red staining (polarized light, percent of total tissue surface area occupied by birefringence) in costal diaphragm sections of 1m, 3m and 6m CD and HFD-fed mice. 4 animals per group at each time point. (C) Masson’s trichrome staining of costal diaphragm sections from 6-month CD vs HFD-fed mice. Demonstrates deposition of collagen (blue) around intramuscular adipocytes (closed arrowheads) more prominent in HFD-fed mice; while perivascular collagen (open arrowheads) is also prominent in CD-fed mice. Representative images from 4 animals per group. Scale 25 µm. (D) Correlation plot showing relationship between polymerized collagen area (percent cross-sectional surface area occupied by birefringence) and measured specific force in costal diaphragm strips subjected to isometric force testing. n = 6 CD and 7 HFD strips from animals at 6m time point. (E-I) Relative mRNA expression of transcripts encoding collagen species, collagen processing proteins and mediators of tissue fibrosis in whole costal diaphragms of 6m CD and HFD-fed mice. n = 5-9 animals per group: (E) type 1 collagen (Col1a1), type 3 collagen (Col3), type 4 collagen (Col4), type 6 collagen (Col6), (F) lysyl oxidase (Lox), (G) matrix metalloproteinase (MMP)-2 (Mmp2), MMP-9 (Mmp9), MMP-14 (Mmp14), (H) transforming growth factor (TGF)-β1 (Tgfb1), TGF-β3 (Tgfb3), (I) platelet-derived growth factor (PDGF)-AA (Pdgfa), fibroblast growth factor (FGF)-2 (Fgf2), connective tissue growth factor (CTGF) (Ctgf), thrombospondin (TSP)-1 (Thbs1). Data
analysis employed Student’s t-test for 2-group, single variable comparisons; 2-way ANOVA (with Sidak’s post hoc test) for 2-variable comparisons; and linear regression for correlation testing. *p <0.05, **p<0.01, ***p<0.001.

**Figure 4.** Fibroadipogenic progenitors give rise to diaphragmatic adipocytes and collagen-producing cells, while proliferating with DIO. (A) PDGFRα Cre-mT/mG mouse model employed for lineage tracing of cells derived from PDGFRα⁺ precursors. (B) Confocal whole-mount images of unstained diaphragms from 6-month CD and HFD-fed PDGFRα Cre-mT/mG mice. Representative images from 4 animals per group. Scale = 50 µm. (C) Immunostaining for type I and VI collagen (COL1 and COL6, respectively) in whole-mount costal diaphragm samples from the same animals. Note deposition of COL1 near and GFP⁺ cells, and distribution of COL6 surrounding clusters of adipocytes. Scale = 100 µm. (D) Platelet-derived growth factor (PDGFR)α (Pdgfra) mRNA levels in whole costal diaphragms of wild type C57Bl/6 mice fed CD or HFD for 6-months. n = 4 CD and 5 HFD-fed animals. (E) Flow cytometry plots showing percent GFP⁺/CD31⁻/CD45⁻/integrin α7⁻ nucleated cells (FAPs) among total nucleated cells isolated from costal diaphragms of 6-month CD and HFD-fed Sca-1 GFP mice. Representative plots (each showing cells from the costal diaphragm of a single animal) from 4 unique experiments. Table shows cell cycle analysis (employing DAPI signal) performed on the FAPs from each group. Data analysis employed Student’s t-test. *p < 0.05

**Figure 5.** Isolated FAPs demonstrate obesity-induced fibrotic gene expression signature, with increased ex vivo proliferation and collagen deposition. (A) Relative mRNA levels in Lin (CD31 and 45)⁻/ Sca1⁺ stromal cells (FAPs) and Lin⁺/Sca-1⁻ stromal cells (non-FAPs).
isolated from costal diaphragms of mice fed control diet (CD) or high fat diet (HFD) for 6 months (6m). Each sample consists of pooled stromal isolate from 3 CD or 2 HFD-fed animals. Each group contains 4 samples. Transcripts analyzed are those encoding PDGFRα (Pdgfra), Pax-7 (Pax7), type I collagen (Colla1), type III collagen (Col3), type VI collagen (Col6), THBS-1 (Thbs1) and TGF-β3 (Tgfb3). (B) Ki67 staining of isolated FAPs from mice fed CD or HFD for 6m cultured in standard medium with 10% FBS. Phalloidin GFP counter-stain, DAPI nuclear stain. Representative images of 3 unique experiments, each comparing 3 CD and 2 HFD-fed mice. Scale 25 µm. Bar graph indicates quantification of Ki67+ nuclei/total nuclei in 10 10x fields per group in each experiment. (C) Type I collagen immunocytochemistry in FAPs isolated from 6m CD and HFD-fed mice and cultured in standard medium with 10% FBS. Representative images of 3 unique experiments, each including 3 CD and 2 HFD-fed mice. Quantification of relative surface area occupied by type I collagen in 10 10x fields per group in each experiment (normalized to cell number). (D) Gene expression profiling in Lin-/Sca1+ FAPs from 6m CD and HFD-fed mice. Transcripts analyzed include those encoding PPARγ2 (Pparg2), TCF-21 (Tcf21), T-box (TBX)-15 (Tbx15), CEBPα (Cepba), PGC1a (Pgc1a), UCP-1 (Ucp1) and IL-6 (Il6). (E) BODIPY staining in FAPs isolated from 6m CD and HFD-fed mice and subjected to adipogenic differentiation protocol. Representative images of 3 unique experiments, each including 3 CD and 2 HFD-fed mice. Quantification of 10 10 x fields per group per experiment. (F) Western blot for THBS-1 in plasma of 6m CD and HFD-fed mice. n = 3 CD and 4 HFD-fed mice. (G) Proliferation of FAPs isolated from 6m CD and HFD-fed mice cultured in medium containing 1% FBS supplemented with 0, 1 or 5 µg/mL THBS-1. Proliferation calculation based on percentage of Ki67+ nuclei/ total nuclei in 10 10x fields per group per experiment. Based on 3 unique experiments each employing 3 CD and 2 HFD-fed mice. Data analysis employed
Student’s t-test for 2-group comparisons, 1-way ANOVA (with Tukey post hoc test) for >2-group comparison, 2-way ANOVA (with Sidak post hoc test) for 2-variables. *p <0.05, **p<0.01, ***p<0.001.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
| Experiment              | Target, animal, conjugate, company, catalog # | Dilution | Target, animal, conjugate, company, cat # | Dilution |
|-------------------------|-----------------------------------------------|----------|------------------------------------------|----------|
| **Tissue IHC**          | Perilipin, rabbit, Cell Signaling, D418       | 1:100    | Goat anti-rabbit IgG, biotin, Vector, BA-1000 | 1:250    |
|                         | UCP-1, rabbit, Alpha Diagnostics, UCP11-A     | 1:100    |                                          |          |
|                         | F4/80, rat, Abcam, ab6640                    | 1:100    | Donkey anti-rat IgG, Alexa Flour 594, Invitrogen, A21209 | 1:250    |
| **Flow Cytometry**      | CD31, rat, PE, eBioscience, 12-0311-81        | 1:500    |                                          | ---      |
|                         | CD45, rat, PE, eBioscience, 12-0451-82        | 1:500    |                                          | ---      |
|                         | Integrin α7, rat, PE, Invitrogen, MA5-23608   | 1:500    |                                          | ---      |
|                         | PDGFRα, rat, APC, Molecular Probes, A18382    | 1:1000   |                                          | ---      |
| **Whole mount IHC, Culture ICC** | Type I collagen, rabbit Rockland, 600-401-103 | 1:100    | Goat anti-rabbit, Alexa Fluor 647, Jackson Immunoresearch, 111-605-144 | 1:250    |
|                         | Type VI collagen, rabbit, Rockland, 600-401-108| 1:100    |                                          |          |
|                         | Ki67, rabbit, Abcam, ab15580                 | 1:250    |                                          |          |

**Table S1. Antibodies.** Immunohistochemistry (IHC), Immunocytochemistry (ICC)
| Gene   | Forward primer sequence | Reverse primer sequence | Probe sequence                      |
|--------|-------------------------|-------------------------|-------------------------------------|
| 36B4   | TGACATCTTTAAACCCCG     | TTCCATCCAGTTGCTTCT     | ---                                 |
| 36B4   | TTATACCCITGAAITCCCG    | CGGCTGGTACCATTGATG     | /FAM/AGGCCCTGC/ZEN/ACTCTGCTT       |
| Adiposq| CTCTCCTGTTCTCTTTAAATC | ACCAAAGAACCTGCAATC    | ---                                 |
| Adipin | GTTCACCTCTTGTCTCTGTA  | TGATGTGACAGGTGATG     | /FAM/AAAGCTATCC/ZEN/CAGAAATGCTGTTG |
| Ap2    | GACAAGATGACAGATGACAT  | GTCAGCCTTCTATAACACAT   | /FAM/ACTCTTTC/ZEN/CATCCACTCTGAC    |
| Cebpa  | AGAAGTCCTGAGACAAGACAGCA | GCGTTTGTTGCTTTATCTGAGTC | ---                                 |
| Col1a1 | CATTGTTATACGTCACTGAC   | CGCAAAAGCATCTACATGTCTAGG | /FAM/CCTGCCAGTGT/SCAAAGCTGAACA   |
| Col5a1 | CTTCTGTTCTCTGCTGTC    | CAAACCTTCTACTATCC     | ---                                 |
| Col4   | ATTTCTGGTCCCTCCTCTC   | GCTCTCAAGTCTCTGCTTTC  | ---                                 |
| Col6   | AAGTGCTGTAGGCACATGCTC | CCAGATGAGTGAGATGACCTG | ---                                 |
| Cigf   | GTCGACTGAGGAAAAACATTAA | CCAGAAAGCTCAAACATTGACAG | ---                                 |
| Enol   | CTTGGCTATGGGTCTCCAGTC | GCAGGGAAGAGAGATTTATGCTG | ---                                 |
| Fgfi2  | GAAACACTCTCTCTGTAAACACACTTT | GTCAAACTACAACCTCAAGCA   | ---                                 |
| Il1b   | GAAACAGTCTCTCTCTTCTGCC | CGACCAATCTCTATTGCAGAAGTT | ---                                 |
| Il6    | AACCCGTAGCTAAGTAAAGATAAAGTTGAGT | GGGAGGCTTTCTGGAATTGACTATGCTTC | ---                                 |
| Lep    | CATTGCTATACGAAAGAC    | ATCTTTGGAACAATCAGAAGTG | ---                                 |
| Lox    | CAAGAGACATCGGACTTTCTTAC | TGGCCATCAAGAGCTCATAG    | ---                                 |
| Lpl    | CATTGTCTCTCTTCTTGTAC   | TGTATGAGGAAACTTGTAGGG  | ---                                 |
| Mmp2   | GTGCAGCTGCTAAGATGATGT  | TCCACACCATCAACATTGGA   | ---                                 |
| Mmp9   | AAGATGCTGATAATGTTGGG  | ATAGCTCTGCTCTGACTG     | ---                                 |
| Mmp14  | GCCGCTTTTCAATACATAG   | GCAACTCACTCGCCGCAA     | /FAM/CAACGTCA/ZEN/CCCAGTCACTTTC  |
| Pax7   | GAAGAGCAACACAGCATC    | GTGCTTCTATCTCATGCAGCC  | ---                                 |
| Pdgfa  | CAAAGAATCTCTCTCTTACTCAG | GATACCTGCGCTATGCTTG     | ---                                 |
| Pdgfra | TTAGAAGAGTGTGGAACTGCTCT | ATTCCTTCTGCTGACATTGAC  | ---                                 |
| Pgc1a  | CTATGAGCTATGAGCGCAGA   | ATAGCTGTCTTCTATCCG    | ---                                 |
| Pnarsy | CTGTTGAGCTAGTCTATAGCC | TGTTTATGTTTGAAGAACTTGGG | /FAM/CCATGCTCT/SCGAGGAAACATGGAAT |
| Thh15  | TAAGAGATGAAAGAAAGCCGCAAAG | TTGAGAGCTCCTACATCATGC   | ---                                 |
| Tcf21  | AGGTCATCACTTGTGCTTC   | GCTCATGCTCATTAAGGC     | ---                                 |
| Tgfb1  | CGGATGTCTGACAGTATGGAAGA | GCGGACATCTATGCTAAAGAGG  | ---                                 |
| Tgfb3  | ACTGGAGACATAGGAAGACA    | GCGAAGAGATCATAAATTTCCGAC  | ---                                 |
| Thbs1  | AAAAAAACATTCTCCGACAATTTCC | CTATCTGACTCTGACCAAGCT   | ---                                 |
| Tnfa   | TCACGTGTTGAGGTTGCTTAC   | TGACTTCTGAGTTGATG      | ---                                 |
| Ucp1   | GCATCGAGGACAAATCAGC    | GCCACACTCTGCATTAAG    | ---                                 |

Table S2. QPCR primers
SUPPLEMENTAL FIGURES

Figure S1. (A) Glucose tolerance test (1g/kg intraperitoneal) in 6-month CD and HFD-fed mice. n = 5 animals per group. (B) Diagram of mouse diaphragm showing muscular components (costal and crural diaphragm), central tendon and anatomic landmarks (esophagus, aorta and inferior vena cava). Green box indicates prototypical strip from costal diaphragm isolated for \textit{ex vivo} testing. Black dashed line indicates region from which sagittal paraffin sections were obtained and orientation of the sectioning plane. (C) \textit{Ex vivo} contractile testing apparatus. Image shows a costal diaphragm strip suspended in Ringer’s bath electrically stimulated through platinum electrodes. Red arrowhead indicates intact rib attachment (sutured to servomotor). Yellow arrowhead indicates free central tendon edge (sutured to force transducer). (D) Correlation plot demonstrating relationship between diaphragm excursion amplitude (measured on non-invasive ultrasound) and specific force (measured on isolated costal diaphragm strips) in 6-month CD and HFD-fed mice. n = 5 CD and 6 HFD-fed animals. Each specific force value represents mean of measurements taken from 2 strips per animal. (E) Correlation plot demonstrating relationship between total body weight and specific force in 6-month CD and HFD-fed animals. n = 5 CD and 6 HFD-fed animals. (F) Correlation plot demonstrating relationship between inguinal adipose depot weight (1 depot per animal) and specific force in 6-month CD and HFD-fed animals. n = 5 CD and 6 HFD-fed animals. (G-I) Resting tension (RT) (dyn), time to peak tension (TTPT) (ms) and half relaxation time (1/2) RT (ms) measured on diaphragm strips of 6-month CD and HFD-fed mice n = 5 CD and 6 HFD-fed mice. Each point represents mean value of measurements.
taken from 2 strips per animal. (J) H/E stained paraffin sections from costal diaphragms of 6-month CD and HFD-fed mice. There are no appreciable differences in myofiber morphology. Representative images from 4 animals per group. Scale = 50 µm. (K) Diaphragm thickness measured on sagittal costal diaphragm sections from 1-month (1m), 3-month (3m) and 6-month (6m) CD and HFD-fed mice. 10 measurements per 10x field, 5-10 fields per section, 3 non-consecutive sections per animal, 4 animals per group. No significant difference between groups at any time point. Within both CD and HFD-fed group, thickness at 6m time point was significantly greater versus 1m time point. (L) Percent of muscle cross sectional area occupied by myofibers in CD and HFD-fed mice at 1m, 3m and 6m time points. (M) Average cross-sectional surface area (SA) of individual myofibers in sagittal costal diaphragm sections from 6-month CD and HFD-fed mice (µm²). Data analysis employed Student’s t-test for 2-group comparisons, 1-way ANOVA (with Tukey post hoc test) for >2-group comparisons, 2-way ANOVA (with Sidak post hoc test) for 2 variables, and linear regression for correlation analysis. *p<0.05

**Figure S2.** (A) Body weights of 16-week-old wild type (WT) C57BL/6 and ob/ob mice fed control diet n = 5 CD and 7 ob/ob mice. (B-F) Diaphragm motion parameters measured on aforementioned mice: (B) Amplitude (mm). (C) Inspiratory velocity (mm/s). (D) Expiratory velocity (mm/s). (E) Peak to peak time (ms). (F) Duty cycle (inspiratory duration/peak to peak) (percent). (G) Specific force (kN/m²) measured on 2-4 mm-wide costal diaphragm strips isolated from WT and ob/ob mice n = 5 mice per group. Each point represents mean value of measurements taken from 2 strips per animal. (H) H/E-stained sagittal sections of WT and ob/ob costal diaphragms, Representative images from 5 animals per group. Arrowheads indicate clusters of intramuscular adipocytes, present in both groups. Scale 100 µm. (I) Average size (cross-sectional surface area) of individual costal diaphragm adipocytes (µm²) in WT and ob/ob mice. n = 5 mice per group. (J) Number of costal diaphragm adipocytes per mm² cross-sectional tissue surface area in WT and ob/ob mice. n = 5 mice per group. Statistical analysis employed Student’s t-test. * p <0.05, ** p<0.01, ***p<0.001, ****p<0.0001.
Figure S3. (A) Relative mRNA expression of transcripts encoding interleukin (IL)-1β (*Il1b*), tumor necrosis factor (TNF)α (*Tnfa*), IL-6 (*Il6*) and F4/80 (*Emr1*) in whole costal diaphragm samples from control diet (CD) and high fat diet (HFD)-fed mice at 6-month time point. n = 9 animals per group. (B) F4/80 immunohistochemistry in costal diaphragm samples from CD and HFD-fed mice at 6-month time point. There are few F4/80+ macrophages in either group, and no crown-like structures like those visible in gonadal adipose tissue (GAT) collected from the same HFD-fed animals. White and yellow arrowheads indicate clusters of adipocytes, respectively in phase and fluorescence images. Red and light blue arrowheads respectively indicate crown-like structures in phase and fluorescence images. Representative images of 4 animals per group. Scale 50 µm. Statistical analysis employed Student’s t-test. ** p<0.01.

Fig. S4. Sirius red-stained costal diaphragm sections from WT and *ob/ob* mice viewed under bright field and polarized light. Scale = 100 µm. Bar graph indicates quantification of sirius red staining (polarized light, percent of total tissue surface area occupied by birefringence) in costal diaphragm sections (excluding collagen of epimysium and surrounding large blood vessels). Representative images of and quantification from 3 non-consecutive sections from 5 CD and HFD-fed mice. Statistical analysis employed Student’s t-test.
Figure S5. Unstained whole mount costal diaphragm images from mT/mG and PDGFRα Cre-mT/mG mice viewed with confocal microscopy. (A) Both images show areas containing large clusters of adipocytes. These are unlabeled in the mT/mG (Cre') sample, but GFP⁺ in the PDGFRα Cre-mT/mG sample. Scale = 25 µm. (B) Absence of staining over the epimysium of the mT/mG diaphragm with rabbit IgG (negative control) antibody. Scale = 50 µm. (C) Prominent type I collagen staining in the epimysium of the mT/mG costal diaphragm. Scale = 50 µm. (D) Robust type VI collagen staining at the central tendon junction of the mT/mG diaphragm. Scale = 50 µm.
Figure S6. (A) Flow cytometry gating scheme for analysis of FAPs from stromal cell isolates of costal diaphragms from *Sca-1 GFP* mice. Lin− diaphragm-derived cells, i.e., negative for CD31 and CD45 (left), were used to enrich (Sca-1) GFP+ FAPs (middle). (Sca-1) GFP+ FAPs are >90% co-positive for PDGFRα (right). Representative plots, each showing an individual animal 4 unique experiments. (B) Negative control staining for CD31/CD45/integrin α7 antibody cocktail (left). Negative GFP signal in CD31−/CD45−/integrin α7− cells from a wild type C57BL/6 mouse (middle). Negative control for PDGFRα staining (right) (C) Cell cycle analysis (based on DAPI signal) of CD31−/CD45−/integrin α7−/(Sca-1) GFP+ stromal cells isolated from costal diaphragms of 6-month CD and HFD-fed *Sca-1 GFP* mice. Representative plots each showing an individual animal from 4 unique experiments.
Figure S7. (A-B) Ex vivo analysis of costal diaphragm stromal cells isolated from CD and HFD-fed PDGFRα Cre-mT/mG mice at the 6-month time point (B) Ki67 nuclear staining of stromal cells. Bar graph indicates percent Ki67+ among DAPI+ nuclei of GFP+ cells. n = 3 20x fields taken of isolates from 3 animals per group. (B) Immunocytochemistry for type I collagen (COL1) and type VI collagen (COL6) in non-permeabilized stromal isolates from the same animals. Representative images from 3 20x fields taken of isolates from 3 animals per group. Statistical analysis employed Student’s t-test. **p<0.01.