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ORIGINAL PAPER

# Leptin is a physiological regulator of skeletal muscle angiogenesis and is locally produced by PDGFRα and PDGFRβ expressing perivascular cells

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## Abstract

Skeletal muscle capillarity is characteristically reduced in mature leptin receptor-deficient (*Lepr*$^{db}$) mice, which has been attributed to the capillary loss that occurs secondary to metabolic dysfunction. Despite wide recognition of leptin as a pro-angiogenic molecule, the contribution of this adipokine has largely been overlooked in peripheral tissues. Moreover, prior documentation of leptin production within skeletal muscle indicates a potential paracrine role in maintaining local tissue homeostasis. Thus, we hypothesized that leptin is a physiological local paracrine regulator of skeletal muscle angiogenesis and that its production may be modulated by nutrient availability. *Lepr*$^{db}$ mice exhibited impaired angiogenesis during normal developmental maturation of skeletal myocytes, corresponding with an inability to increase vascular endothelial growth factor-A (VEGFA) mRNA and protein levels between 4 and 13 weeks. In cultured murine and human skeletal myocytes, recombinant leptin increased VEGFA mRNA levels. Leptin mRNA was detectable in skeletal muscle, increasing with prolonged high-fat feeding in mice, and with adiposity in human subjects. Platelet-derived growth factor receptor (PDGFR) α− and PDGFRβ− expressing perivascular cell populations were identified as leptin producing within skeletal muscle of mice and humans. Furthermore, in response to 2 weeks of high-fat feeding, PDGFRβ+ but not PDGFRα+ cells increased leptin production. We conclude that leptin is a physiological regulator of the capillary network in skeletal muscle and stimulates VEGFA production by skeletal myocytes. PDGFRβ expressing perivascular cells exhibit the capacity to act as local "nutrient-sensors" that couple nutrient status to leptin production in skeletal muscle.

**Keywords** Leptin · Angiogenesis · VEGFA · Pericyte

Thomas Gustafsson and Tara L. Haas have equally contributed to this work.

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## Introduction

Obesity-related metabolic disorders increase an individual's risk for adverse health outcomes (i.e., type-2 diabetes mellitus, cardiovascular diseases) and have become a large economic burden for healthcare systems worldwide. A key determinant of the extent of metabolic disturbance that occurs with obesity is an individual's capacity to effectively regulate energy homeostasis [1]. Leptin is an adipocyte-derived polypeptide hormone with established roles in the control of energy homeostasis, including regulation of food intake and energy expenditure, through its actions on the central nervous system [2]. Importantly, leptin receptors are ubiquitously expressed in peripheral tissues, facilitating the high functional pleiotropism of this hormone (i.e., metabolism, wound healing, bone remodeling, and reproduction) [3]. In skeletal muscle, leptin inhibits lipogenic pathways

and promotes the oxidation of fatty acids [4, 5]. The resulting decrease in skeletal muscle triglyceride content can consequently protect against the development of insulin resistance in mice fed a high-fat (HF) diet [4, 6]. Moreover, leptin exhibits direct insulin-sensitizing effects on skeletal muscle via regulation of the insulin growth factor binding protein-2 [7], indicating co-operation of leptin and insulin signaling within skeletal muscle.

In addition to the metabolic role of leptin within skeletal muscle, leptin is recognized as a pro-angiogenic molecule [8–11], widely studied within the contexts of wound healing [12, 13] and cancer progression [14–16]. Angiogenesis, the growth of new capillaries from pre-existing ones, is a crucial tissue adaptation that occurs locally in response to the particular metabolic demands of that tissue to ensure adequate oxygenation and exchange of nutrients and waste [17, 18]. However, a pro-angiogenic role for leptin within skeletal muscle, and resultant implications for energy homeostasis, remains to be investigated.

Several pieces of evidence implicate leptin in the regulation of skeletal muscle capillarization. Capillary number is typically reduced in skeletal muscle of leptin-resistant rodents that carry a point mutation within the leptin receptor gene [19–22]. Although this deficit has been proposed to occur secondary to the metabolic dysfunction that is characteristic of leptin resistance, it is noteworthy that metabolic dysfunction induced by prolonged high-fat feeding in wild-type mice does not alter, or even increases, skeletal muscle capillarization [23, 24]. These divergent outcomes suggest that deficiency in leptin signaling, rather than metabolic dysfunction per se, contributes directly to the underlying reduction in skeletal muscle capillary number. Importantly, leptin mRNA and protein have previously detected within skeletal muscle [25], indicating that it may act as a local paracrine signaling molecule. However, the cellular source and regulation of local leptin production in skeletal muscle are still unknown. Thus, we hypothesized that leptin is a physiological local paracrine regulator of skeletal muscle angiogenesis and that local leptin production is modified in accordance to its biological role to maintain local energy homeostasis.

## Methods

### Animal experiments

Male homozygous leptin-resistant mice on a C57BL/6J background (B6.BKS(D)-*Leprdb*/*J*; referred to as *Lepr*$^{db}$) and age-matched wild-type C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA) and used for experiments at 13 weeks of age. Four-week-old *Lepr*$^{db}$ mice were generated by crossing *Lepr*$^{db}$/*Lepr*$^{+}$ mice and genotype was confirmed by PCR analysis (http://www.jax.org). For diet comparisons, male C57BL/6J mice fed either normal chow (NC) or high-fat (HF) diet (D12329 and D12429, respectively; Research Diets, Inc., New Brunswick, NJ, USA) for 9 weeks. A separate group of 8-week-old male C57BL/6NCrl mice (Charles River), placed on similar NC or HF diets (#D12329 and #D12331) for 2 weeks, were used to assess the cellular source of leptin in isolated cellular components from skeletal muscle (described below). For the assessment of *leptin* mRNA in skeletal muscle homogenates, measurements were conducted on tissue collected from a prior study, in which male FVB/N mice were fed either NC or HF diets (D12329 and D12331, respectively) for 16 weeks [23].

### Human studies

To measure leptin expression in myoblast, myotubes and skeletal muscle tissue biopsies from *vastus lateralis* muscle of healthy non-smoking young men were collected using the percutaneous needle biopsy technique. From the same individual, *LEPTIN* mRNA could be analyzed in these two myogenic maturation stages and in the individual's whole skeletal muscle tissue [26]. Effects of leptin treatment were assessed in cultured myoblasts/myotubes (described below). To establish the distribution of leptin by immunostaining, biopsies from *vastus lateralis* muscle of 3 healthy non-smoking young men were collected using the percutaneous needle biopsy technique, cleaned of connective tissue and frozen in liquid nitrogen cooled isopentane for use in histological assessments [27]. The mean (range) age, height, and weight of volunteers were 25 (24–27) year, 179 (173–187) cm, and 73 (70–74) kg, respectively. Biopsies from vastus lateralis of sedentary overweight individuals (a subset of the META-PREDICT HIT trial) [28], taken at the pre-training time-point, were utilized for RNA analysis of *LEPTIN* and *GAPDH*. Subjects' mean (range) age, height, weight, and %body fat are as follows: 39 (22–50) year, 170 (155–191) cm, 93.9 (69.6–139) kg, and 41.9 (23.9–54.9)%.

### Skeletal myocyte insulin sensitivity

Ex vivo insulin stimulation of the *extensor hallucis proprius* (EHP) muscle of *Lepr*$^{db}$ and WT mice was performed as previously described [23]. Briefly, muscles were removed from both legs and pre-incubated in HEPES-saline buffer at 37 °C. After 30 min, insulin (25 mU/mL, Humalog) was added to one of each muscle pair for an additional 30 min (37 °C), then muscles were snap frozen in liquid nitrogen for subsequent analysis of phosphorylated Akt and Akt levels by Western blotting analysis.

## Protein analysis

Protein extraction and western blot analysis were performed as previously described [29]. Primary antibodies were pSer473 Akt and Akt (#4058 and #9272; Cell Signalling Technology, Pickering, ON, Canada). Secondary Antibody was goat anti-rabbit IgG-horseradish peroxidase (#111-035-003, Jackson ImmunoResearch Laboratories, WestGrove, PA, USA). Densitometry analysis was performed with Carestream software (Molecular Bioimaging, Bend, OR, USA). VEGFA protein was assessed in gastrocnemius muscle homogenates [100 µg protein in PBS using a mouse VEGFA ELISA (DY493-05; R&D Systems, Minneapolis, MN, USA)].

## Cell culture experiments

C2C12 myoblasts were cultured on gelatin-coated flasks with DMEM (11960-044; Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada), 50 µg/mL penicillin/streptomycin, 1% Glutamax (35050-061, Invitrogen), and 1 mM sodium pyruvate (Invitrogen). C2C12 myoblasts were seeded to confluence on 12-well tissue culture dishes and treated for 4 h with either 100 nM recombinant leptin (AF498; R&D Systems, Minneapolis, MN, USA) or 25 mU/mL insulin (Humalog), then processed for RNA analysis. Human myoblasts were isolated from healthy individuals as previously described [30] and cultured on collagen-coated flasks with proliferation media consisting of DMEM/F-12 (11330-032; GIBCO) supplemented with 20% FBS, 50 µg/mL penicillin/streptomycin, and 1 mM sodium pyruvate. Human myotubes were differentiated in a 12-well tissue culture dish by seeding myoblasts to ~ 50% confluency and switching to differentiation media (containing only 2% FBS) for 5 days before leptin (100 nM) and insulin (25 mU/mL) treatments and RNA analysis. Human umbilical vein endothelial cells (HUVECs) (C-003-5C; Life Technologies, Carlsbad, CA) were cultured in Medium 200 (M200) supplemented with low-serum growth supplement, and 1% antibiotic–antimycotic (Life Technologies). When confluent, cells were harvested in TRIzol (Life Technologies) prior to RNA extraction.

## Histological assessments

Transverse cryosections (10 µm thick) were cut from the mid-belly of the mouse *plantaris* (PLA) muscle to assess capillary-to-fiber ratio (C:F). Capillaries were detected using FITC-*Griffonia simplicifolia* isolectin B4 (1:100; Vector Laboratories, Burlington, ON, Canada) and distinguished from arterioles by co-staining with Cy3-anti-α-smooth muscle actin (SMA) antibody (1:300; C6198; Sigma-Aldrich, Oakville, ON, Canada). Digital images were captured using an Axiovert 200 M microscope (×20 objective; Zeiss, Oberkochen, Germany) equipped with a cooled digital CCD camera and Metamorph software. Quantification of C:F was made by a blinded observer from 3 to 4 non-overlapping fields of view per muscle.

Cellular localization of leptin was visualized in paraformaldehyde-fixed transverse sections (10 µm) of the soleus muscle in mice or *vastus lateralis* human biopsies using a rabbit anti-leptin antibody (1:200, #16227, Abcam) diluted in blocking buffer (5% donkey serum + 0.05% Triton X-100 in PBS). For co-localization, leptin-positive immunoreactivity was co-stained with the following goat antibodies: anti-mouse PDGFRα (1:100, #AF1026), anti-mouse PDGFRβ (1: 100, #AF1042), anti-human PDGFRα (1:100, #AF-307-NA), or anti-human PDGFRβ (1:100, #AF385, R&D systems). Intramuscular lipids were detected with Alexa Fluor488 BODIPY (0.25 µg/mL,#D3922, ThermoFisher Scientific) and capillaries were visualized in mouse and human sections using Dylight 649-*Simplicifolia* isolectin $B_4$ (1:50, #DL-1208) and Ulex *Europaeus* Agglutinin (1:50, #DL-1068, Vector Laboratories), respectively. Alexa Fluor488 donkey anti-rabbit (#705-165-147) and Cy3 conjugated donkey anti-goat (#711-545-152) cross-adsorbed secondary antibodies (1:400; Jackson Immunoresearch) were used as appropriate. Specificity of secondary antibodies was confirmed with secondary only negative controls (Supplementary Fig. S1). All images were captured with a Zeiss LSM700 inverted confocal microscope equipped with either a EC PLAN-Neofluar 40×/1.30 Oil M27 or a Plan-Apochromat 63×/1.40 Oil DIC M27 objective using identical gain and exposure settings across samples.

## RNA analyses

For murine skeletal muscle, total RNA was isolated from the gastrocnemius muscle using RNeasy mini-kit (Qiagen, Inc. Toronto, ON, Canada) and 150 ng of total RNA was reversed transcribed into cDNA. For human skeletal muscle, total RNA was extracted from vastus lateralis biopsies using TRIzol (Invitrogen, Carlsbad, CA). The integrity of total RNA was confirmed by 1% agarose gel electrophoresis. RNA was reversed transcribed with Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Carlsbad, CA) and random hexamer primers (Roche Diagnostics, Mannheim, Germany) in a total volume of 20 µL. For most cell culture experiments, cDNA was generated using Cells-to-cDNA II lysis buffer followed by reverse transcription (ThermoFisher Scientific, Burlington, ON, Canada). For HUVECs, RNA was extracted using the TRIzol method and was reversed transcribed using the high-capacity cDNA reverse transcription kit (LifeTechnologies). Analyses were performed using TaqMan real-time quantitative PCR on the Rotor-Gene Q PCR platform (Qiagen, location) using Mastermix (4444963; ThermoFisher Scientific) and TaqMan

FAM-labeled probe/primer sets, as follows: mouse; *Vegfa* (Mm00437306_m1), *Thbs1* (Mm00449032_m1), *Leptin* (Mm00434759_m1), *Cspg4* (Mm00507257_m1), *Pdgfra* (Mm00440701_m1), *Pdgfrb* (Mm00435546_m1), *Zfp423* (Mm01246261_m1), *Cebpα* (Mm00514283_s1), *Pparg* (Mm00440940_m1) and *Hprt1* (Mm00446968_m1) and human; *VEGFA* (Hs00900055_m1), *LEPTIN* (Hs00174877_m1), *HPRT1* (Hs01003267_m1) and *GAPDH* (4352934E). Vic labeled mouse *Gapdh* probe (#4308316) and primer sets (#4304106, Abcam) were used in some experiments. mRNA was quantified relative to appropriate housekeeping genes and expressed as $2^{-\Delta C_t}$.

### Isolation of microvascular fragments, PDGFRα+ and PDGFRβ+ cells from murine skeletal muscle

Microvascular fragments were isolated from mice fed a NC or HF diet for 2 or 9 weeks using CD144 (#555289, BD Biosciences)-coupled dynabeads (#14311D, ThermoFisher Scientific) following a brief digestion of minced skeletal muscles from the lower and upper hindlimbs (30 min at 37 °C; 0.05% Type-II collagenase). In mice fed a NC or HF diet for 2 weeks, PDGFRα+ and PDGFRβ+ cells were isolated using dynabeads conjugated to CD140a (#135902) and CD140b (#136002, BioLegend) antibodies, respectively, following collagenase digest (2 h) and filtration through a 100-µm cell strainer. All antibody incubations were performed at 4 °C for 20 min and selection performed using a magnet. Isolated cellular fractions were immediately lysed with Trizol, and RNA was purified using the RNeasy micro-kit (Qiagen, Inc. Toronto, ON, Canada). RNA analysis was performed as described above. PDGFRα+ and PDGFRβ+ cell populations were assessed by qPCR analysis of *Pdgfra* and *Pdgfrb* transcripts in the respective cell isolates.

### Statistical analysis

Data are presented as means ± SEM of biological replicates. All statistical analyses were performed with Prism 5 (GraphPad Software, La Jolla, CA, USA) and were parametric and two-tailed. Significance was established at $P < 0.05$, by unpaired Student's *t* test or 2-way ANOVA with TUKEY post hoc analysis as appropriate.

## Results

### Impaired angiogenesis and suppressed VEGFA production during post-natal skeletal muscle growth of *Lepr$^{db}$* mice

To discriminate the influence of metabolic dysfunction from leptin resistance on skeletal muscle capillarity, histological assessments of skeletal muscles from *Lepr$^{db}$* mice were performed at 2 different ages: 4 weeks, to coincide with the onset of metabolic disturbances and 13 weeks, to represent a stage of chronic metabolic impairment (Fig. 1a). *Lepr$^{db}$* mice had a lower capillary-to-fiber ratio (C:F) in the plantaris muscle compared to wild-type (WT) controls at both 4 and 13 weeks of age (Fig. 1b). Wild-type mice exhibited a 30% higher C:F at 13 weeks compared to 4 weeks of age (1.87 ± 0.04 vs. 1.43 ± 0.04, respectively; $P < 0.05$) but there was no significant increase in *Lepr$^{db}$* mice over the same time (1.38 ± 0.05 vs. 1.17 ± 0.1; NS) (Fig. 1b). Capillary density (CD) also was significantly reduced in *Lepr$^{db}$* mice at 13 weeks compared to 4 weeks (Fig. 1c). Enlargement of myocyte cross-sectional area (CSA) with age was blunted in *Lepr$^{db}$* mice, resulting in smaller myocyte CSA at 13 weeks relative to WT mice (Fig. 1d), consistent with a previous report [31]. Together, these data indicate that muscle growth during the post-weaning period is not matched sufficiently with capillary growth in *Lepr$^{db}$* mice.

To determine if the difference in muscle capillarization was associated with dysregulation in the gene expression of major angiogenic regulators in *Lepr$^{db}$* mice, the skeletal muscle levels of thrombospondin-1 (THBS1) and vascular endothelial growth factor-A (VEGFA) were measured. In both age groups, levels of *Thbs1* mRNA in *Lepr$^{db}$* mice were unaltered compared to WT mice, but a genotype-independent decline in *Thbs1* mRNA was observed at 13 weeks (Fig. 1e). At 13 weeks, VEGFA transcripts (Fig. 1f) and protein (Fig. 1g) were significantly increased in WT mice compared to their respective 4-week age group. However, VEGFA mRNA and protein levels did not increase between 4 and 13 weeks in *Lepr$^{db}$* mice, and were significantly lower than WT mice at 13 weeks (Fig. 1g, h). These data suggest that leptin signaling is essential to the age-related increase in *Vegfa* expression in skeletal muscle, and its absence in *Lepr$^{db}$* mice leads to impaired post-natal angiogenesis.

### Leptin increase VEGFA mRNA levels in murine and human skeletal muscle cells

To directly assess the influence of leptin on VEGFA levels, in vitro experiments were performed using skeletal muscle cells, as these cells constitute ~90% of VEGFA production within skeletal muscle [32]. Leptin treatment (100 nM) significantly increased VEGFA transcripts not only in murine C2C12 myoblasts (Fig. 2a), but also in primary human myoblasts (Fig. 2b) and differentiated human myotubes (Fig. 2c), demonstrating that leptin is a positive regulator of VEGFA levels in skeletal myocytes. Because leptin signaling converges with the insulin signaling pathway, and insulin has also been implicated in the regulation

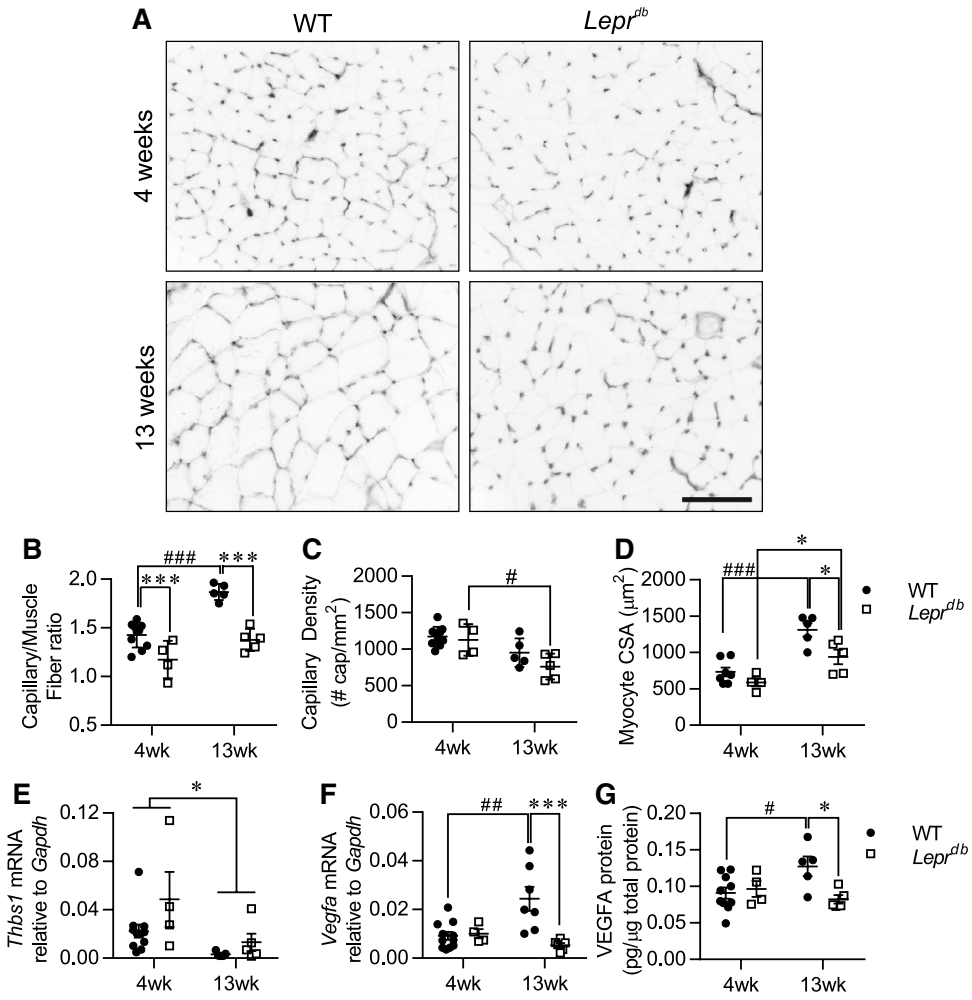


**Fig. 1** Skeletal muscle characteristics of *Lepr*$^{db}$ and WT mice at 4 and 13 weeks of age. **a** Representative images of the plantaris muscle stained with *G. simplicifolia* lectin-FITC to visualize capillaries. Grayscale images were inverted to enhance visualization of capillaries. Scale bar = 100 μm. Quantifications of **b** capillary/muscle fiber ratio, **c** capillary density, and **d** myocyte cross-sectional area (CSA) were performed from at least 3 independent fields of view per mouse. **e** *Thbs1* and **f** *Vegfa* mRNA in the gastrocnemius muscle was assessed by real-time quantitative PCR relative to *Gapdh* and expressed as $2^{-\Delta C_t}$. **g** VEGFA protein levels were assessed by Elisa using gastrocnemius muscle homogenates. $^{*}P<0.05$, and $^{***}P<0.001$ vs. age-matched WT mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. corresponding 4-week-old mice, with Tukey post hoc analysis

of VEGFA gene expression in several cell types [33–35], we investigated whether disruptions to insulin signaling in *Lepr*$^{db}$ mice may also regulate VEGFA levels within skeletal muscle. VEGFA transcripts were significantly increased in C2C12 myoblasts (Fig. 2d), as well as in human myoblasts (Fig. 2e) and differentiated myotubes (Fig. 2f) after 4 h of insulin treatment. To specifically assess skeletal myocyte insulin sensitivity, we performed ex vivo insulin stimulation of the mouse *extensor hallucis proprius* (EHP) muscle and found that insulin-induced phosphorylation of Akt was impaired significantly in 13-week-old *Lepr*$^{db}$ mice relative to WT controls (Fig. 2h, i). Together, these data indicate that skeletal myocyte insulin sensitivity may also contribute to reduced skeletal muscle VEGFA levels in *Lepr*$^{db}$ mice.

## Local skeletal muscle leptin production is increased with obesity

Angiogenesis generally is regulated at the tissue level in response to local metabolic demands [17], thus we tested for leptin expression within skeletal muscle. Leptin transcripts were detectable in both murine and human skeletal muscle (Fig. 3a, b). Leptin mRNA was elevated in mice fed HF diet for 16 weeks (Fig. 3a) and in human subjects with a higher body fat percentage (Fig. 3b), demonstrating that local leptin production increases in skeletal muscle with obesity. However, leptin transcripts were not detectable in C2C12 myoblasts and were expressed at negligible levels ($Ct > 35$) in human myoblasts and differentiated myotubes, indicating that leptin production within skeletal muscle does not originate from satellite cells or skeletal myocytes.

We used immunostaining to gain insight into the cellular localization of leptin within skeletal muscle. Since adipocytes are the predominant cellular producer of leptin [36], leptin-positive immunoreactivity was confirmed first in adipose tissue, finding leptin localized within the thin cytoplasmic rim of individual adipocytes (Fig. 4a). In mouse skeletal muscle, leptin immunoreactivity was detected faintly within the peri-myocyte interstitium as well as distinctly in focal interstitial sites that appeared more numerous and intense with HF feeding (Fig. 4a). To evaluate potential

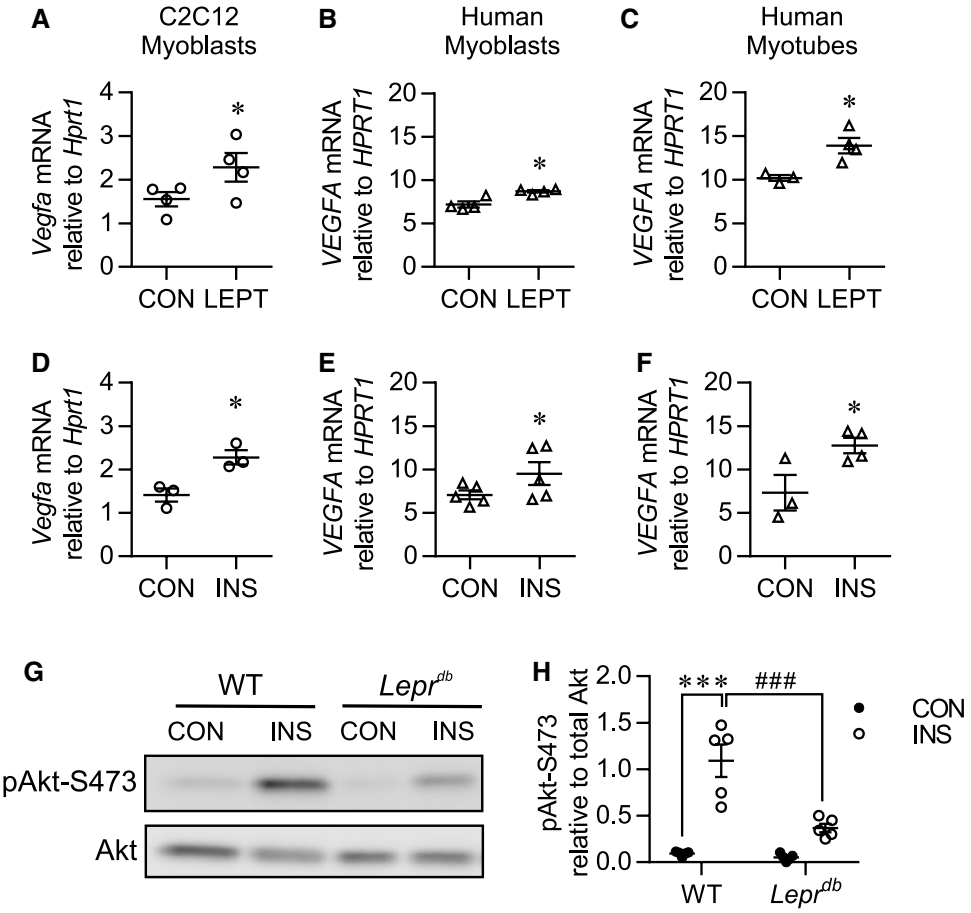


**Fig. 2** Leptin increases VEGFA mRNA expression in murine and human skeletal muscle cells. **a, d** C2C12 and **b, e** human myoblasts as well as **c, f** differentiated myotubes were stimulated with either **a–c** 100 nM of recombinant leptin (LEPT) or **d–f** 25 mU/mL of insulin (INS) for 4 h. *Vegfa* mRNA levels were assessed by quantitative PCR relative to *Hprt*1 and expressed as $2^{-\Delta C_t}$. $^{*}P < 0.05$ vs. unstimulated control (CON) group. **g, h** *Extensor hallucis proprius* muscles were isolated from WT and *Lepr*$^{db}$ mice and analyzed for insulin sensitivity. pSer473-Akt was assessed following ex vivo incubation in the absence or presence of insulin and normalized to total Akt levels. $^{***}P < 0.001$ vs. WT control muscles. $^{\#\#\#}P < 0.001$ vs. WT insulin stimulated muscle, with Tukey post hoc analysis

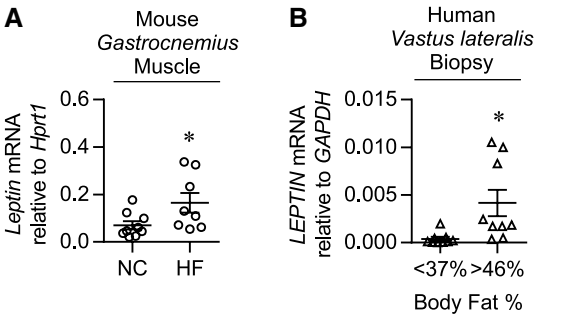


**Fig. 3** Leptin is produced within skeletal muscle. *Leptin* mRNA levels were assessed as a measure of leptin production in the gastrocnemius muscles of (**a**) mice fed a normal chow (NC) or high-fat (HF) diet for 16-weeks, and (**b**) in biopsies collected from human subjects stratified by body fat percentage. Leptin mRNA levels were assessed by real-time quantitative PCR relative to *Hprt1* or *GAPDH* and expressed as $2^{-\Delta C_t}$. $^{*}P < 0.05$ vs. respective NC group

co-localization of leptin immunoreactivity and skeletal muscle-resident adipocytes, BODIPY was utilized to demarcate lipid-laden cells. There were no identifiable BODIPY-positive interstitial cells observed in murine muscle sections, and a complete lack of overlap between BODIPY staining and leptin immunoreactivity. Human muscle biopsies were characterized by the substantial presence of intramyocyte lipid droplets. Similar to mouse muscles, there were no identifiable BODIPY-positive interstitial cells and no overlap between BODIPY staining and leptin-positive immunoreactivity (Fig. 4b). Due to the focal interstitial locale of leptin staining, co-localization with capillary structures was next examined. The majority of leptin immunoreactivity co-localized with lectin-positive capillaries in both mouse and human muscles (Fig. 5a). In microvascular fragments isolated from murine skeletal muscle, leptin transcripts were detected and increased with 9 weeks of HF feeding (Fig. 5b). However, neither cultured murine skeletal muscle endothelial cells nor human umbilical vein endothelial cells had detectable levels of leptin mRNA (Ct > 35). All together, these data indicate that perivascular cells in close proximity to capillaries provide a local source of leptin within skeletal muscle.

## PDGFRα+ and PDGFRβ+ cells are leptin producing within skeletal muscle

PDGFRα+ and PDGFRβ+ cells are relatively abundant in the perivascular space and can exhibit adipogenic potential [37, 38] leading us to examine their potential contribution

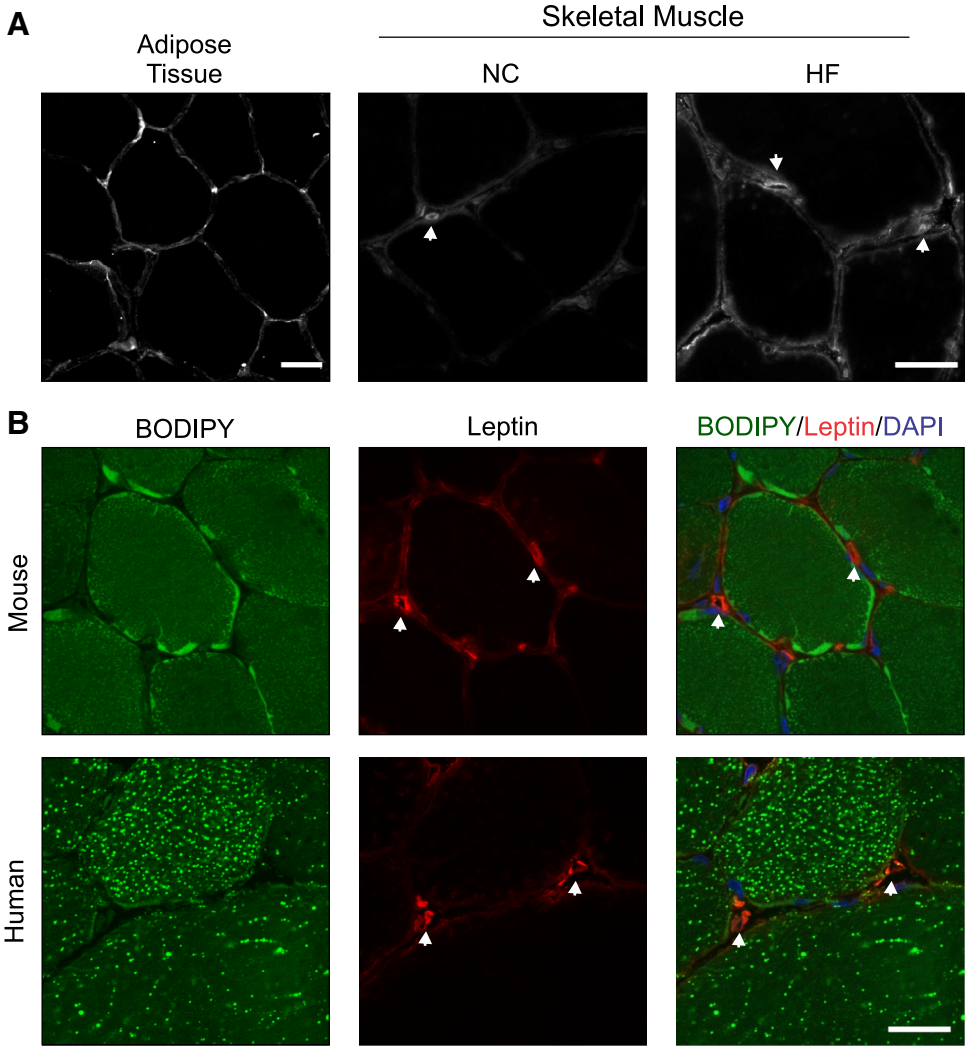


**Fig. 4** Cellular localization of leptin within skeletal muscle. **a** Leptin-positive immunoreactivity (arrows) in adipose tissue sections (positive control) and soleus muscle sections from NC- and HF-fed mice. **b** Co-staining for BODIPY (to detect lipids) and leptin in murine soleus muscle and human biopsy sections. Scale bar = 20 µm

to local leptin production. In both murine and human skeletal muscles, PDGFRα immunoreactivity was detectable at discrete locations in the skeletal muscle interstitium and importantly, in close proximity to lectin staining (capillaries) (Fig. 6a). The majority of PDGFRα staining demonstrated overlap with leptin immunoreactivity (Fig. 6b). This overlap was evident particularly around capillaries, but also occasionally in cells within the skeletal muscle interstitium. PDGFRβ immunoreactivity was associated exclusively with microvessels (Fig. 7a), and co-localized prominently with leptin immunoreactivity in both mouse and human muscles (Fig. 7b).

To specifically assess leptin production in PDGFRα+ and PDGFRβ+ cells, a second group of mice was fed a HF diet for 2 weeks and leptin mRNA was measured in multiple cell populations isolated following collagenase digestion of skeletal muscles (Fig. 8a). In this cohort, leptin transcripts were detected in the digested muscle fraction and enriched in isolated capillary fragments (Fig. 8b). Importantly, capillary fragments expressed both *Pdgfra* and *Pdgfrb* transcripts (Fig. 8c), confirming the presence of these cell populations. Individual populations of PDGFRα+ and PDGFRβ+ cells were isolated following a longer collagenase digest to generate a single-cell suspension (Fig. 8d) and *leptin* mRNA was assessed in both PDGFRα+ and PDGFRβ+ cells. While *Leptin* mRNA was detectable in the PDGFRα+ cell population from all NC-fed mice, only 1 out of 5 mice had detectable levels of *Leptin* mRNA in isolated PDGFRβ+ cells (Fig. 8e, f). Interestingly, in response to HF diet, only PDGFRβ+ cells exhibited an increased expression of *Leptin* transcripts (Fig. 8e, f). To further characterize the identity and adipogenic commitment of PDGFRα+ and PDGFRβ+ cells, we assessed several additional markers. PDGFRα+ and PDGFRβ+ cells expressed similar levels of the gene product *Cspg4* (Fig. 8e, f), which encodes the pericyte marker NG2. Both PDGFRα+ and PDGFRβ+ cells had detectable mRNA levels of the pre-adipocyte commitment factors, zinc finger protein 423 (*Zfp423*) and Ccaat enhancer binding protein

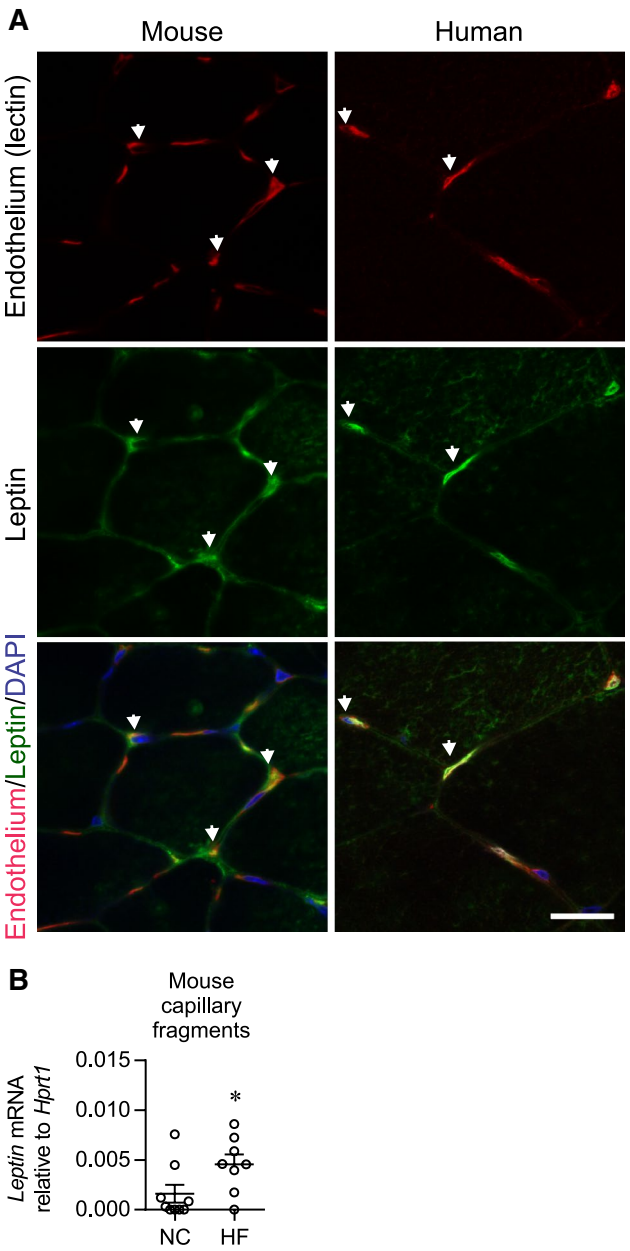


**Fig. 5** Leptin is produced in skeletal muscle capillary fragments. **a** Histological assessment of mouse and human muscle sections demonstrating overlap between leptin immunoreactivity and lectin-positive (capillary) staining (arrowheads). Scale bar = 20 µm. **b** Leptin mRNA levels were assessed in capillary fragments isolated from skeletal muscles of NC- and HF-fed mice by quantitative PCR relative to *Hprt1* and expressed as $2^{-\Delta C_t}$. $^*P < 0.05$ vs. respective NC mice

alpha (*C/ebpa*), whereas mRNA encoding the mature adipocyte marker peroxisome proliferator-activated receptor γ (*Pparg*) was undetectable (Fig. 8e, f). Notably, HF feeding further increased levels of *Zfp423* transcripts in isolated PDGFRβ+, but not PDGFRα+ cells (Fig. 8e, f), implicating HF diet as a trigger for a population of PDGFRβ+ cells to undergo adipogenic commitment.

## Discussion

In this study, we provide evidence that leptin is a physiological regulator of the capillary network in skeletal muscle. Leptin resistance, induced by genetic mutation of the leptin receptor, was associated with a reduction of skeletal muscle capillarity as early as 4 weeks of age, which was exacerbated by an impaired angiogenic response during normal developmental maturation in the post-weaning period. Leptin was demonstrated to function as a positive regulator of skeletal myocyte VEGFA production in both mice and humans. Notably, local leptin production in murine and human skeletal muscle increased with obesity, consistent with the role of leptin as a sentinel of nutrient status. Furthermore, we identified perivascular PDGFRα+ and PDGFRβ+ cells as local sources of leptin in murine skeletal muscle. The increased production of leptin within PDGFRβ+ cells in response to a short-term HF diet implicates these cells as components of a local signaling network associated with nutrient excess.

A common explanation for the lower skeletal muscle capillarity associated with leptin-deficiency models of obesity is that a loss of existing capillaries (rarefaction) occurs as a result of chronic metabolic dysfunction that is characteristic of leptin deficiency [39]. However, these inferences are often based on cross-sectional data collected from a single cohort of adult mice, limiting the interpretation of microvascular remodeling events that occur as a result of leptin deficiency. In the current study, capillary number (C:F) was unaltered in *Lepr*$^{db}$ mice between 4 and 13 weeks, in contrast to the increase in capillary number that occurred in WT mice over the same time period, in parallel with the age-associated increase in muscle size. These findings indicate that the lower capillary density in mature *Lepr*$^{db}$ mice cannot be explained by capillary rarefaction. Rather, our data are consistent with the idea that impaired capillary growth in *Lepr*$^{db}$ mice underlies the reduction of capillary density in *Lepr*$^{db}$ mice, reflecting an uncoupling of capillary growth from myocyte enlargement during normal developmental maturation. It is noteworthy that *Lepr*$^{db}$ mice exhibited a substantially lower C:F before a sustained period of metabolic dysfunction, suggesting that the lower capillary number associated with leptin resistance occurs independently of metabolic impairments. This idea is strengthened by reports that metabolic dysfunction induced by a prolonged HF in leptin-sensitive mice is not associated with a decline in capillary number, but instead results in unaltered or increased capillary number [23, 24]. While the relationship between metabolic dysfunction and skeletal muscle capillarity is complex, a growing number of studies have shown a positive correlation between skeletal muscle capillarity and insulin sensitivity [40, 41], and the present findings provide support for the concept that the lower skeletal muscle capillarity in

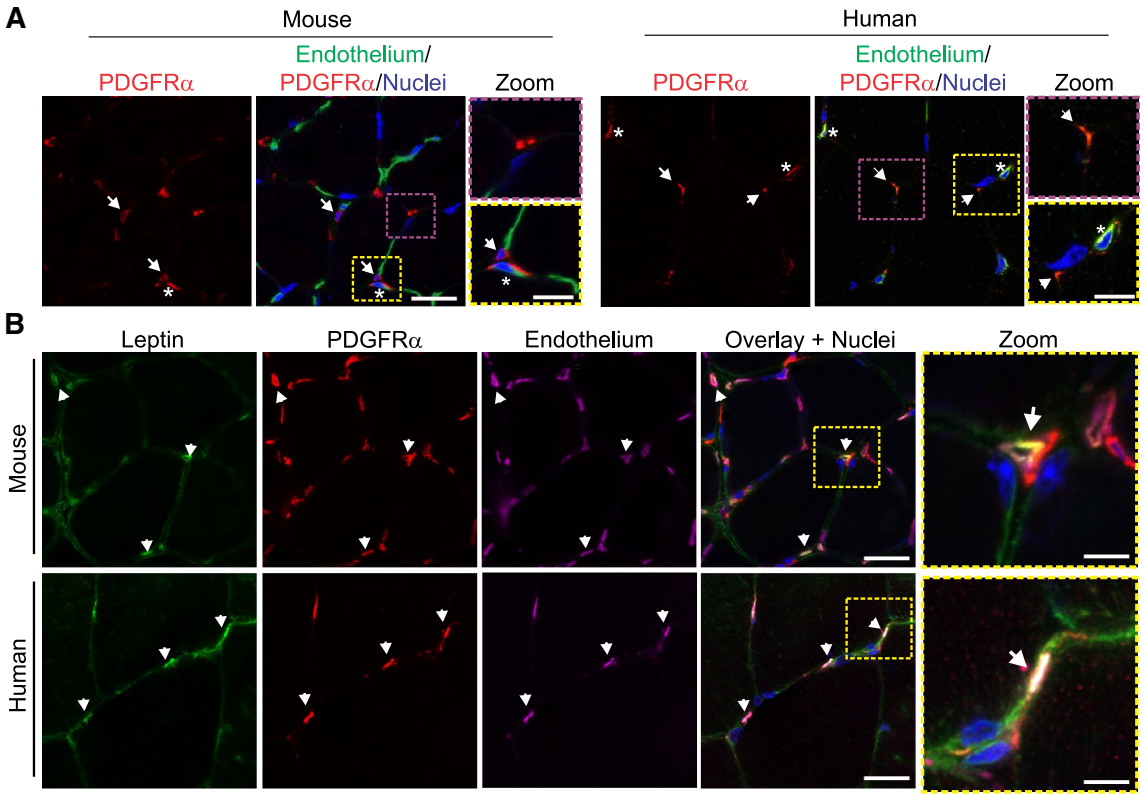


**Fig. 6** Co-localization of PDGFRα and leptin immunoreactivity. **a** Immunostaining for PDGFRα and lectin in mouse and human skeletal muscle in order to determine the cellular localization of PDGFRα+ cells. PDGFRα+ cells were identifiable in the interstitium (arrows) and associated with lectin + capillaries (asterisks). **b** Co-staining for leptin (green), PDGFRα (red), lectin (magenta), and DAPI (blue) demonstrating overlap between leptin and a subset of PDGFRα+ cells (arrows) in both mouse and human muscles. Scale bar = 20 µm (5 µm for zoomed images)

*Lepr*$^{db}$ mice contributes to the development of metabolic dysfunction.

Leptin has been shown to directly enhance endothelial cell proliferation and the formation of capillary networks in various in vitro and in vivo angiogenic assays [9–11, 42, 43]. Nonetheless, in light of the critical role of VEGFA in skeletal muscle angiogenesis [32, 44, 45], the observed failure of *Lepr*$^{db}$ mice to increase VEGFA in skeletal muscle undoubtedly contributes to the poor angiogenic capacity observed in these mice. Suppressed VEGFA production in *Lepr*$^{db}$ mice at 13 weeks is unlikely a direct consequence of metabolic dysfunction, considering that VEGFA levels increase in skeletal muscle of HF-fed WT mice [23, 24]. Instead, our results implicate leptin resistance to be the cause of suppressed VEGFA production, since recombinant leptin induced VEGFA expression in cultured murine myoblasts and primary human myocytes. This finding is consistent with prior reports that leptin is a direct regulator of VEGFA production in tumors [46, 47]. VEGFA production in murine and human myocyte cultures was also positively regulated by treatment with insulin. This is noteworthy since leptin can sensitize insulin signaling [7] and *Lepr*$^{db}$ mice exhibited impaired skeletal myocyte insulin sensitivity. Thus, convergence between leptin and insulin signaling may contribute to the overall reduction in VEGFA levels in the skeletal muscle of mature *Lepr*$^{db}$ mice. Overall, these findings demonstrate that leptin functions as a pro-angiogenic factor within skeletal muscle by promoting VEGFA production in skeletal myocytes.

Since the control of angiogenesis is fundamentally linked with the local tissue environment [17, 18], the current detection of leptin transcripts, within human skeletal muscle as well as in rodent muscle, is pivotal in positioning leptin as a plausible regulatory factor in this process. Moreover, the increase in local leptin production observed in response to HF feeding in mice and with adiposity in human subjects suggests that local leptin may act as a pro-angiogenic molecule in obesity by stimulating skeletal myocyte VEGFA production. Expansion of the capillary network in this context may contribute to the maintenance of metabolic homeostasis by increasing oxygen supply and the surface area for metabolic exchange, thus supporting the increased reliance

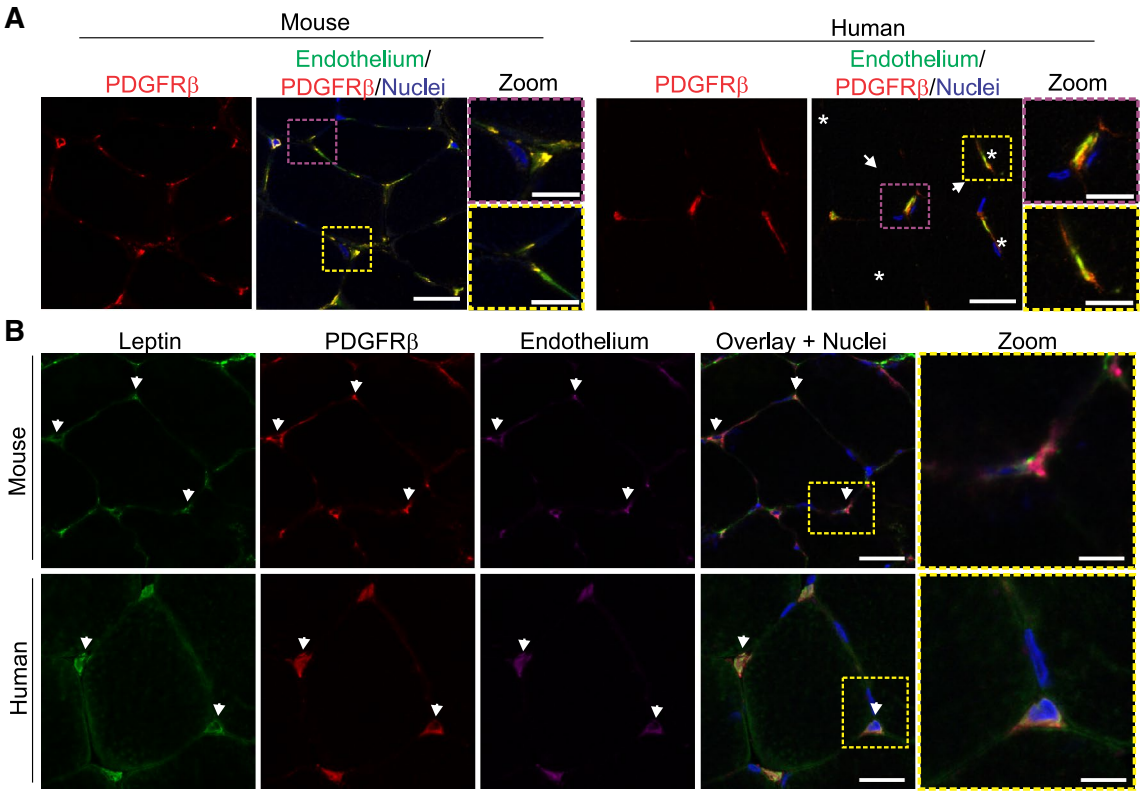


**Fig. 7** Co-localization of PDGFRβ and leptin immunoreactivity. **a** Immunostaining for PDGFRβ and lectin (capillaries) in mouse and human skeletal muscle demonstrating the perivascular localization of PDGFRβ+ cells. **b** Leptin (green), PDGFRβ (red), lectin (magenta), and DAPI (blue) demonstrating overlap between leptin and pericapillary PDGFRβ+ cells (arrows) in both mouse and human skeletal muscle. Scale bar = 20 μm (5 μm for zoomed images)

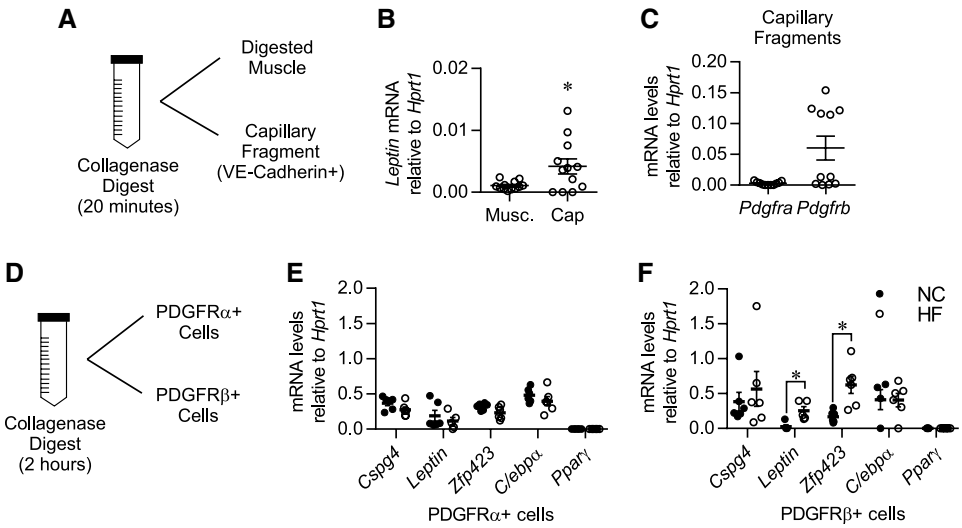


**Fig. 8** PDGFRα+ and PDGFRβ+ perivascular cells are leptin producing within skeletal muscle. Isolation of various cell fractions was performed using skeletal muscles from mice fed a NC or HF diet for 2 weeks. Following a brief collagenase digest, capillary fragments were isolated from the entire muscle digest using VE-Cadherin (VECAD) conjugated magnetic beads (**a**) and *leptin* mRNA was measured (**b**) demonstrating enrichment in *leptin* transcripts in the isolated capillary fragments. Relative levels of *Pdgfra* and *Pdgfrb* mRNA were also assessed in capillary fragments (**c**). Following a longer digest, PDGFRα- and PDGFRβ-positive cells were isolated using antibody coupled magnetic beads (**d**). *Leptin* mRNA and transcript identifiers of pericytes and adipocyte lineage were quantified in the PDGFRα+ (**e**) and PDGFRβ+ (**f**) cells and expressed as $2^{-\Delta C_t}$. $^{**}P < 0.01$ vs. NC group

on lipid metabolism in skeletal muscle. The role of local leptin does require further investigation since serum leptin levels are also characteristically elevated with nutrient excess and it is a challenge experimentally to assess the relative contributions of locally produced and circulating leptin. Nonetheless, it should be noted that skeletal muscle uptake of circulating leptin is relatively low [48, 49], which implies that local leptin production may exert a significant influence on skeletal myocyte cellular processes that have previously been attributed to circulating leptin, including the regulation of energy expenditure [50] and lipid oxidation [4, 5].

Leptin transcripts were detectable in PDGFRα- and in PDGFRβ-positive cells. Identification of the cellular source of leptin within skeletal muscle is important to be able to establish a foundation for mapping its potential paracrine signaling pathways. PDGFRα and in PDGFRβ cell surface markers are typical (but not exclusive) of interstitial cells that exhibit adipogenic capacity (i.e., fibroadipocyte precursors) and pericytes, respectively [38, 51]. Notably, the pattern of PDGFRβ immunoreactivity in both mouse and human skeletal muscle appeared most consistent with that of capillary-associated pericytes, while PDGFRα reactivity was found in association with capillaries as well as at non-capillary locations. Interestingly, a subset of pericytes possessing adipogenic potential also expresses PDGFRα [38], which is consistent with the detection of pericyte marker *Cspg4* in both PDGFRα+ and PDGFRβ+ populations. This suggests that leptin-producing pericytes may be both PDGFRα+ and PDGFRβ+, but the antibody-based selection method used in the current study to isolate these cells did not resolve the extent of co-expression of PDGFRα and PDGFRβ. Thus, the available data lead us to conclude that local leptin production within skeletal muscle originates dominantly from a population of pericytes, although a contribution of interstitial PDGFRα+ cells cannot be excluded.

The presence of *Zfp423* transcripts in both PDGFRα+ and PDGFRβ+ cells is consistent with commitment to an adipogenic lineage [52]. Several reports indicate that pericytes exhibit multipotent capability to differentiate into adipogenic and myogenic lineages [38] and they were reported to produce leptin in the liver [53]. Thus, the present findings are in accordance with the current knowledge that leptin transcripts are predominantly produced by mature adipocytes [54], but can also be induced in adipogenic precursor cells [55]. Leptin production in PDGFRβ+ cells did increase following HF feeding, coinciding with an increased expression of *Zfp423*, which implies that exposure to a HF diet provokes adipocyte commitment within the pericyte population. However, terminal differentiation of these cells to adipocytes is unlikely under the conditions of our study, considering the negligible levels of *Pparg* and the lack of detectable adipocytes in skeletal muscle by histology. Thus, local leptin production within skeletal muscle seems to be closely linked to the adipogenic potential rather than terminal differentiation of precursor cells. Responsiveness of PDGFRβ+ cells may be facilitated by their perivascular localization, as they are situated in a privileged position for exposure to fatty acids as they are transported across the capillary to the skeletal myocytes. Taking these observations into consideration, increased leptin production by pericytes in response to HF feeding may serve as a mechanism to communicate systemic nutrient status within skeletal muscle in order to coordinate a tissue-level response to nutrient excess.

In conclusion, through dual investigation of murine and human muscle, the current study identifies leptin as a stimulus for VEGFA production by skeletal myocytes and demonstrates a novel role for leptin as a physiological regulator of the skeletal muscle capillary network. Furthermore, the local production of leptin by peri-capillary PDGFRα+ and PDGFRβ+ adipogenic precursors points to pericytes as plausible "nutrient-sensors" in both rodent and human skeletal muscle. Finally, considering leptin's role as a rheostat for energy homeostasis, local leptin production may serve as an intrinsic mechanism that facilitates intercellular communication of energy status within skeletal muscle, in part through promotion of capillary expansion. In sum, our findings caution against the interpretation of peripheral consequences of impaired leptin signaling in rodents as driven solely by systemic metabolic disturbance and draws attention to the physiological relevance of leptin paracrine actions within skeletal muscle.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interests.

**Ethical approval** Animal studies were approved by the York University Committee on Animal Care (#2017-19R3, #2017-20R3) and performed in accordance with the American Physiological Society's guiding principles in the care and use of animal models. Human studies were approved by the Regional Ethics Review Board in Stockholm (Dnr2006/1232-31/1, 2010/786-31/3, Dnr2012/173-31/3, DNR2012/753-31/2) and performed in accordance with the 1964 Helsinki declaration.

**Informed consent** Informed written consent was obtained from all participants following explanation of the experimental procedures.

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