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Leptin treatment has vasculo-protective effects in lipodystrophic mice

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Lipodystrophy syndromes (LDs) are characterized by loss of adipose tissue, metabolic complications such as dyslipidemia, insulin resistance, and fatty liver disease, as well as accelerated atherosclerosis. As a result of adipose tissue deficiency, the systemic concentration of the adipokine leptin is reduced. A current promising therapeutic option for patients with LD is treatment with recombinant leptin (metreleptin), resulting in reduced risk of mortality. Here, we investigate the effects of leptin on endothelial to mesenchymal transition (EndMT), which impair the functional properties of endothelial cells and promotes atherogenesis in LD. Leptin treatment reduced inflammation and TGF-β2–induced expression of mesenchymal genes and prevented impairment of endothelial barrier function. Treatment of lipodystrophic- and atherosclerosis-prone animals ($Ldlr^{-/-}$; aP2-nSrebp1c-Tg) with leptin reduced macrophage accumulation in atherosclerotic lesions, vascular plaque protrusion, and the number of endothelial cells with mesenchymal gene expression, confirming a reduction in EndMT in LD after leptin treatment. Treatment with leptin inhibited LD-mediated induction of the proatherosclerotic cytokine growth/differentiation factor 15 (*GDF15*). Inhibition of *GDF15* reduced EndMT induction triggered by plasma from patients with LD. Our study reveals that in addition to the effects on adipose tissue function, leptin treatment exerts beneficial effects protecting endothelial function and identity in LD by reducing *GDF15*.

lipodystrophy | leptin | EndMT | atherosclerosis | GDF15

Significance

Lipodystrophy syndromes are characterized by loss of adipose tissue, metabolic complications, and accelerated atherosclerosis. Adipose tissue deficiency results in reduced levels of the adipokine leptin. We investigated the effects of leptin on the functional properties of endothelial cells and atherogenesis in lipodystrophy. Leptin reduced endothelial to mesenchymal transition-induced expression of mesenchymal genes and prevented impairment of endothelial barrier function. Leptin administration in a lipodystrophy and atherosclerosis mouse model reduced plaque protrusion and endothelial cells with mesenchymal gene expression in vascular plaques. The effects were mediated by the growth/differentiation factor 15. The data identify an important role for leptin in controlling endothelial cell function in lipodystrophy syndromes.

Lipodystrophy syndromes (LDs) describe a heterogeneous group of disorders characterized by varying degrees of adipose tissue loss. A number of causal mutations in different genes, such as *AGPAT2*, *BSCL2*, or *CAV1*, which are related to lipid metabolism result in the inability to store triglycerides in adipose tissue, which subsequently accumulate in the liver (1). Paradoxically, the metabolic consequences result in pathologies usually seen in obesity, such as dyslipidemia, hepatic steatosis, insulin resistance, and increased risk for diabetes mellitus and atherosclerosis (2, 3). The severity of the observed pathologies in LD correlates with the degree of loss of adipose tissue (4, 5). The imbalance of adipose tissue proportion is also a characteristic feature of obesity, although in an inverse manner. Mechanistically, both LD and obesity cause dysregulation of adipokines such as leptin and adiponectin (6). Leptin was introduced in 1994 as an appetite-suppressive and energy expenditure-inducing molecule in humans (7). In obesity, circulating leptin levels are high due to central leptin resistance, whereas in LD leptin levels are low due to deficient differentiation, loss of function, or apoptosis of fat cells (8, 9). High and low leptin levels are associated with premature atherosclerosis. The precise mechanisms of how leptin affects cardiovascular functions are not completely understood (10, 11). We hypothesize that leptin contributes to obesity-associated acceleration of atherosclerosis by modulating endothelial damage.

The formation of atherosclerotic plaques is a multifactorial process initiated by dysfunction or injury of endothelial cells (ECs). Although ECs are considered fully differentiated cells, they can profoundly change their phenotype and function in response to environmental and mechanical stimuli. Transforming growth factor β2 (TGF-β2) and deleterious conditions such as inflammation, oxidized low-density lipoprotein, or high glucose levels can promote EC transition to a mesenchymal phenotype, a process termed endothelial to mesenchymal transition (EndMT) (12–14). EndMT is associated with a reduction of prototypical endothelial gene expression and impaired EC functions, concomitant with a de novo expression of mesenchymal marker genes. ECs undergoing EndMT lose their prototypical endothelial barrier function, thereby facilitating immune cell infiltration. Phenotype switches of ECs to a mesenchymal phenotype can be found in complex and vulnerable atherosclerotic lesions (15, 16) and thus represent a key event during the development and progression of cardiovascular diseases.

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As leptin dysregulation is one of the key mechanisms in LD (17), we hypothesized that leptin may exert direct effects on ECs. To determine the effects of leptin treatment on endothelial physiology and the development of atherosclerosis in LD, we generated and analyzed a combination of a leptin-deficient lipodystrophic (18) and atherosclerosis-prone mouse model. Our data show that leptin can reverse several parameters of atherosclerosis progression in LD.

Results

Leptin Treatment Reduces Vascular Inflammation and EndMT. Vascular inflammation is an important contributor to the development of atherosclerosis. We first assessed the effect of leptin treatment on the inflammatory response of primary human ECs. Treatment of ECs with interleukin-1β, a proinflammatory cytokine, induced a significant up-regulation of proinflammatory marker genes such as intercellular adhesion molecule 1 (*ICAM-1*) and E-selectin on messenger RNA (mRNA) expression level. Upon additional treatment of ECs with leptin, mRNA levels of these inflammatory markers were significantly decreased in comparison with interleukin-1β treatment on mRNA level (Fig. 1 *A* and *B*). These data demonstrate a reduction of endothelial inflammation by leptin treatment, suggesting that leptin mediates antiinflammatory properties in the endothelium.

Stress stimuli such as inflammation can induce the transdifferentiation of ECs into mesenchymal cell types by the process of EndMT. ECs undergoing EndMT lose their physiological endothelial functions, such as the maintenance of the vascular barrier, and contribute to atherosclerotic plaque formation. We assessed the role of leptin on the process of EndMT, utilizing a TGF-β2–induced EndMT model in primary human ECs. While induced by TGF-β2, cotreatment with leptin inhibited the induction of the mesenchymal gene smooth muscle protein 22-α (*SM22*). As EndMT is a heterogeneous process, we used single-cell sequencing to analyze EndMT (Fig. 1 *C* and *D*). A reduction in ECs expressing mesenchymal markers *SM22*, *SERPINE1*, *FN1*, *VIM*, and *SULF1* was observed following leptin treatment, compared with TGF-β2–treated controls (Fig. 1 *C* and *D*). Consistently, reduced expression of mesenchymal genes *SM22* and calponin (*CNN1*) was observed in bulk EC populations following leptin treatment (Fig. 1 *E* and *F*). Furthermore, immunofluorescence analysis revealed that cotreatment with leptin reduced TGF-β2–mediated induction of SM22 and reversed the loss of the endothelial marker protein VE-cadherin (CDH5) (Fig. 1 *G–I*). To further analyze the effects of leptin on the functional properties of ECs after EndMT, we performed impedance measurements of the EC layer. TGF-β2–treated ECs exhibited reduced resistance, indicating increased permeability after EndMT, which was completely repealed by the addition of leptin (Fig. 1*J*). This was further confirmed by a fluorescence-labeled dextran permeability assay, which showed reduced EC permeability following leptin cotreatment (*SI Appendix*, Fig. S1*A*). Together, these data show that leptin reduces the process of EndMT and thereby protects endothelial barrier functions.

Leptin Treatment Reduces EndMT in an LD Mouse Model. In LD, lipids that are usually stored in fat cells accumulate ectopically in nonadipocytes, resulting in dyslipidemia, fatty liver, and insulin resistance. Thus, patients with LD are at high risk for atherosclerotic cardiovascular diseases. To analyze the effect of leptin treatment on atherogenesis in the setting of LD, we used a leptin-deficient, lipodystrophic mouse model with additional *Ldlr* knockout (*Ldlr*$^{-/-}$; aP2-nSrebp1c-Tg, referred to as LD mice) (Fig. 2*A*). LD animals had increased body weight compared with wild-type mice and elevated levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT), indicating LD-induced impairment of the liver. These changes were repealed by additional treatment with recombinant leptin (Fig. 2 *B* and *C* and Table 1). We found reduced levels of subcutaneous adipose tissue and visceral adipose tissue, increased level of interscapular brown adipose tissue, and increased liver weight in the LD model, although no change in blood glucose level was detected in comparison with the *Ldlr*$^{-/-}$ control mice (Fig. 2 *D* and *E* and Table 1). Serum cholesterol and triglyceride levels increased in LD mice (Fig. 2 *F* and *G*), while treatment with leptin resulted in improved lipid metabolism. In vitro, we found leptin treatment reduced EndMT-induced vascular leakage in ECs. Therefore, we aimed to analyze the effect of leptin treatment on EndMT in LD mice in vivo. EndMT was quantified in atherosclerotic plaques in the aortic root (Fig. 2 *H–K*). The number of ECs ($SM22^{-}CD31^{+}$) did not change (Fig. 2*H*), whereas the numbers of $EndMT^{+}$ ($SM22^{+}CD31^{+}$) (Fig. 2*I*) and mesenchymal cells ($SM22^{+}CD31^{-}$) (Fig. 2 *J* and *K*) per atherosclerotic plaques lesion were significantly increased in LD mice in comparison with control mice, but the numbers decreased after leptin treatment. This suggests that LD mice are more susceptible to atherosclerotic development, which can be reversed by leptin treatment (Fig. 2*L*). During the transition to mesenchymal cells, ECs gain in size (Fig. 1*G*). Accordingly, there was a trend toward a reduced number of cells per atherosclerotic plaque (*SI Appendix*, Fig. S1*B*). Leptin treatment prevented the increase of EndMT in the LD mice as well as the number of mesenchymal cells per plaque. These results reveal the occurrence of EndMT in the pathology of LD and identify a beneficial effect of leptin on EC identity by inhibiting EndMT.

Leptin Treatment Reduces Macrophage Infiltration and Protrusion of Atherosclerotic Plaques in Murine Lipodystrophy. Having established the favorable impact of leptin on EndMT in atherosclerotic plaques, we next analyzed the impact of leptin on macrophage infiltration and plaque protrusion in LD. Interestingly, we found leptin treatment in LD mice reduced the protrusion of atherosclerotic plaques (Fig. 3*A*), which is associated with improved clinical outcomes (19). Macrophage accumulation in atherosclerotic lesions showed a trend toward reduction after leptin treatment in 16-wk-old LD mice. The *Cd68*-positive area per atherosclerotic plaque further increased in 20-wk-old LD mice compared with 16-wk-old LD animals, while leptin treatment reduced the infiltration of macrophages in 20-wk-old mice (Fig. 3 *B* and *C*). There were no differences in the absolute plaque area, collagen content, and lipid content (*SI Appendix*, Fig. S1 *C–E*) of plaques in LD mice at the age of 16 wk when comparing the aortic roots of *Ldlr*$^{-/-}$ and LD mice with leptin or placebo treatment. These data indicate that leptin substitution contributes to the maintenance of the physiological endothelium during LD by inhibiting atherosclerotic progression in terms of macrophage infiltration and plaque protrusion.

Identification of GDF15 as a Leptin-Regulated Signaling Molecule in EndMT and LDs. To identify soluble signaling molecules that are regulated by leptin and presumably mediate the action of leptin in the vasculature, we performed a screening of signaling molecules in the serum of patients with LD via a proximity extension assay (Olink) as well as an expression analysis in ECs after EndMT by single-cell sequencing (Fig. 4 *A* and *B* and Table 2). An intersection analysis of signaling molecules regulated in both LD and EndMT revealed von Willebrand factor (*vWF*), intercellular adhesion molecule 2 (*ICAM-2*), and growth/differentiation factor 15 (*GDF15*) as potentially regulating the downstream effects of

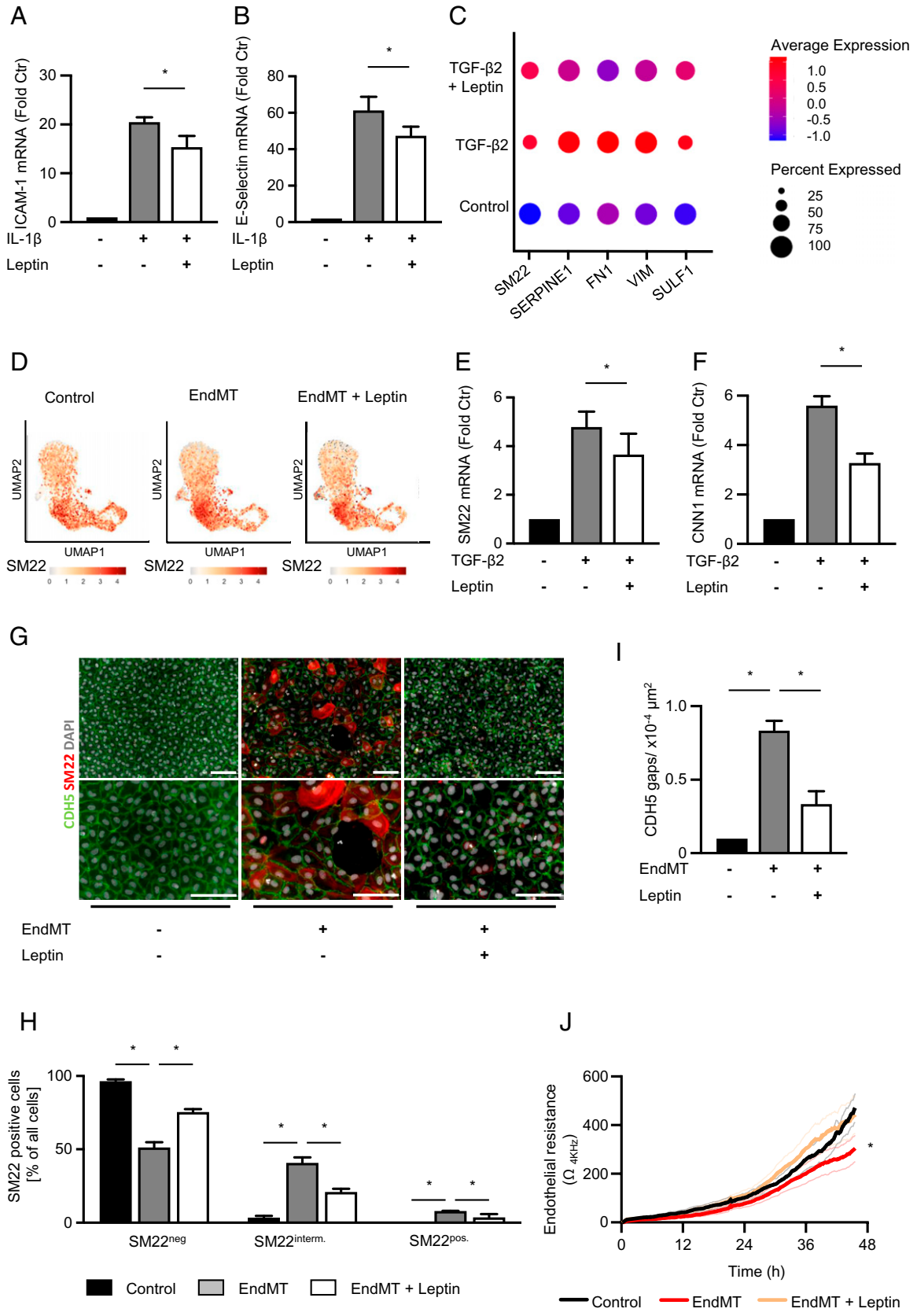

Fig. 1. Leptin reduces endothelial inflammation, EndMT, and vascular leakage. (*A*) mRNA levels of the inflammatory marker *ICAM-1* ($n = 3$) and (*B*) E-selectin in ECs after interleukin-1β and leptin (200 ng/mL) treatment by qPCR, normalized to *RPLP0* (comparative cycle threshold method [$2^{-\Delta Ct}$]) ($n = 4$). (*C* and *D*) *SM22* expression after EndMT induction in EC with leptin treatment, determined by single-cell sequencing. Control cells ($n = 10{,}920$), EndMT ($n = 6{,}743$), and EndMT with leptin treatment ($n = 5{,}647$). (*E*) *SM22* expression ($n = 4$) and (*F*) *CNN1* expression after EndMT with leptin treatment in ECs by RT-qPCR, norm. to *RPLP0* ($2^{-\Delta Ct}$) ($n = 4$). (*G*) Representative immunofluorescence after EndMT with leptin treatment. Nuclei are stained blue, EC marker CDH5 (VE-Cadherin) is stained green, and mesenchymal marker SM22 is stained red. (*H*) EndMT induction with additional treatment with leptin as %SM22-negative, intermediate, and %SM22-positive cells ($n = 3$). (*I*) CDH5 cell gaps per area as vascular leaks after EndMT with leptin treatment ($n = 3$). (*J*) EC resistance after EndMT with leptin ($n = 3$). *$P < 0.05$, Student's *t* test. Ctr, Control.

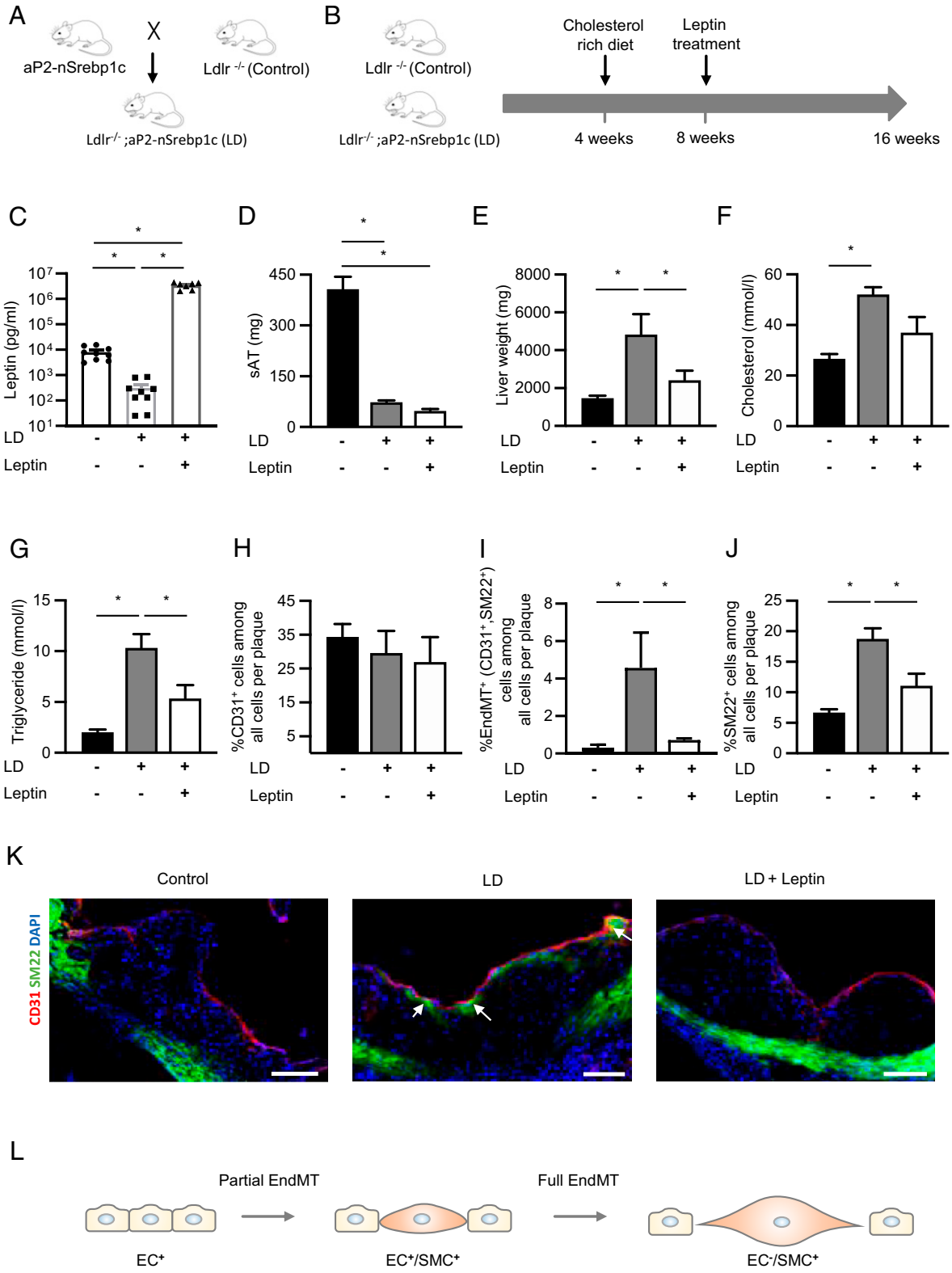

Fig. 2. Leptin treatment reduces EndMT in an LD mouse model. (*A*) Scheme showing generation of the LD model by combining *Ldlr*$^{-/-}$ mice with aP2-nSrebp1c-Tg to obtain atherosclerotic-prone LD mice (*Ldlr*$^{-/-}$; aP2-nSrebp1c-Tg). (*B*) Scheme showing experimental setup of leptin therapy in LD mice. Eight-wk-old (*Ldlr*$^{-/-}$; aP2-nSrebp1c-Tg) male mice were randomized into two groups and treated intraperitoneally with recombinant leptin (3.0 mg/kg body weight [BW]) or saline daily for 8 wk. Non-LD littermates on a *Ldlr*$^{-/-}$ background served as controls. (*C*) Leptin concentration at the end of the treatment. Mice were fasted overnight and the last saline or leptin injection was performed 30 min before sacrifice. Leptin was determined by ELISA in control ($n = 9$), LD ($n = 9$), and LD + leptin ($n = 7$) mice. (*D* and *E*) Weight of subcutaneous adipose tissue (sAT) (*D*) and liver weight (*E*) of control mice and LD mice with leptin (3 mg/kg BW/d for 8 wk) or saline treatment ($n = 3$ to 5). (*F* and *G*) Serum cholesterol (*F*) and triglyceride levels (*G*) ($n = 6$ to 8). (*H*) Number of ECs per atherosclerotic plaques in aortic roots of *Ldlr*$^{-/-}$ control and lipodystrophic *Ldlr*$^{-/-}$; aP2-nSrebp1c-Tg (LD) mice with leptin or placebo treatment. SM22 and CD31 cells in intimal atherosclerotic plaques among all cells per lesion. (*I* and *J*) EC (SM22^{-}CD31^{+}): percentage of EndMT-positive (SM22^{+}CD31^{+}) (*I*) and percentage of mesenchymal cells (SM22^{+}CD31^{-}) (*J*) ($n = 4$ to 6). At least three images from each of at least two spatially separated aortic root sections per mouse were evaluated. Data were averaged per animal. (*K*) Representative pictures of immunofluorescent staining. Arrows indicate EndMT cells. Scale bar, 100 μm. (*L*) Scheme showing transdifferentiation of ECs into mesenchymal cells according to EC and MSC markers. *$P < 0.05$, ANOVA with Tukey post hoc test or Mann-Whitney test.

Table 1. Baseline characteristics of the leptin-treated lipodystrophic mice

	Control*	LD*	LD + leptin*	P	
				C vs. LD	LD vs. LD + leptin†
No.	3–6	4–7	5–8		
Age (wk)	16	16	16		
Sex	Male	Male	Male		
Body weight (g)	28.08 (0.8)	27.9 (0.6)	22.05 (0.8)	0.98	**<0.0001**
Food intake (g/kg/d)‡	153.1 (3.2)	168.4 (1.4)	171.4 (8.9)	0.268	0.928
sAT weight (mg)	407.2 (37)	73.69 (5.0)	47.20 (5.6)	<0.0001	0.59
vAT weight (mg)	682.1 (49)	79.47 (6.5)	72.37 (8.0)	<0.0001	0.97
iBAT weight (mg)	59.54 (4.2)	144.7 (7.0)	115.6 (16)	0.0007	0.21
Liver weight (mg)	1,461 (81)	4,833 (534)	2,411 (226)	0.0004	**0.0016**
Glucose (mmol/L)	8.53 (0.7)	8.46 (0.7)	7.4 (0.5)	0.9966	0.4879
ALAT (μkat/L)	1.29 (0.3)	6.66 (0.6)	3.7 (0.6)	<0.0001	**0.0029**
ASAT (μkat/L)	1.94 (0.3)	6.82 (0.9)	4.23 (0.5)	0.0001	**0.0183**

ALAT, alanine aminotransferase; ASAT, aspartate-aminotransferase; iBAT, intrascapular brown adipose tissue; LD, lipodystrophy syndrome; sAT, subcutaneous adipose tissue; vAT, visceral adipose tissue.
*Data are reported as mean (SEM), ANOVA with Tukey post hoc test.
†*P* values in bold type are statistically significant at <0.05.
‡Food intake is presented as average amount over the whole treatment period (g/kg/mouse/d).

leptin, while GDF15 showed the strongest induction in both conditions, LD and EndMT (Fig. 4*C*). After validation of GDF15 dysregulation in patients with LD ($n = 48$) and control participants ($n = 54$) using enzyme-linked immunosorbent assay (ELISA) (Fig. 4*D* and Table 2), *GDF15* was selected for further analysis based on induction in EndMT and LD. GDF is a member of the TGF-β superfamily whose expression and circulating levels are typically increased in response to cellular stress and disease (20). The increase in *GDF15* during EndMT was inhibited by additional leptin treatment (Fig. 4 *E* and *F*), which was also confirmed in vivo by the protein level in the plasma of LD mice treated with leptin (Fig. 4*G*). Furthermore, leptin substitution reduced the mRNA expression of *GDF15* in the liver and visceral adipose tissue of LD mice (Fig. 4*H* and *SI Appendix*, Fig. S1*F*). Data from the EndMT assay and the in vivo LD mouse model, as well as data from patients with LD, suggest that *GDF15* expression and circulating levels are increased in EndMT and LD, whereas treatment with leptin can abolish this increase. Indeed, leptin and GDF15 are inversely correlated in plasma of mice of the LD model setup ($R = -0.55$; $P = 0.01$) (Fig. 4*I*).

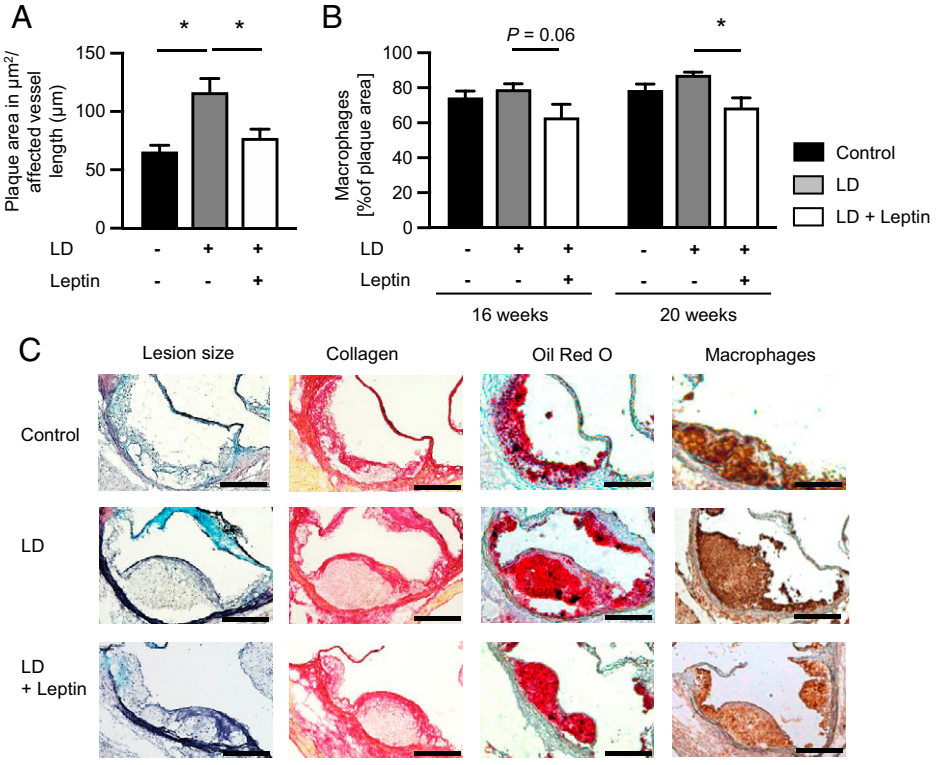

Fig. 3. Leptin treatment reduces atherosclerosis in an LD mouse model. (*A*) Atherosclerotic plaque protrusion was determined by measuring the total plaque area per length of plaque-covered inner vessel wall, determined in sections of vessel cross section ($n = 4$ to 6). (*B*) Bar charts of 16-wk-old animals ($n = 10$) and 20-wk-old mice ($n = 3$ to 6). Data analyzed with ANOVA Tukey post hoc test and Student's *t* test. (*C*) Representative images, Scale bar, 200 μm. Leptin (3 mg/kg body weight) was applied intraperitoneally starting at the age of 8 wk (mean ± SEM). *$P < 0.05$, ANOVA with Tukey post hoc test.

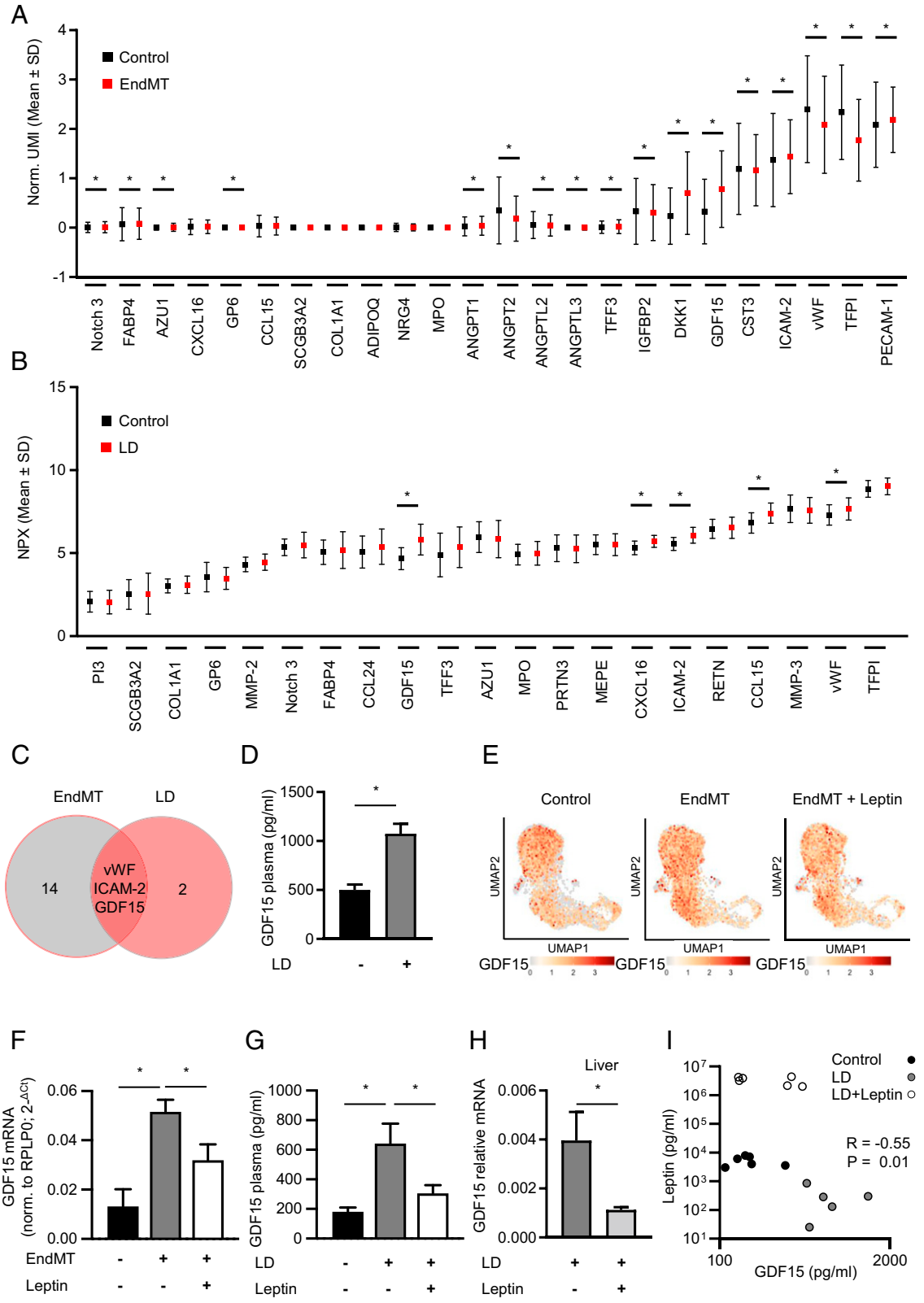

Fig. 4. Leptin treatment reduces EndMT- and LD-mediated *GDF15* induction. (*A*) Expression of signaling proteins after EndMT induction analyzed by single-cell sequencing. Data shown as mean normalized (norm.) unique molecular identifier (UMI) ± SD. *$P < 0.05$. Analyzed control cells (n = 10,920) and cells undergoing EndMT (n = 6,743). (*B*) Expression of proteins in the serum of patients with LD (n = 48) and control participants (n = 54) measured with the Olink assay. Data are shown as mean normalized protein expression (NPX) ± SD for patients with LD (n = 48) and control participants (n = 54). *$P < 0.05$. (*C*) Intersection analysis of regulated cytokines in the serum of patients with LD and after EndMT induction. (*D*) GDF15 level by ELISA in patients with LD (n = 48) and control participants (n = 54). (*E*) *GDF15* expression after EndMT with leptin treatment, determined by single-cell sequencing. Control cells (n = 10,920), EndMT cells (n = 6,743), and EndMT cells with leptin treatment (n = 5,647). (*F*) mRNA levels of *GDF15* (n = 4) in ECs after induction of EndMT with leptin treatment by qPCR, norm. to *RPLP0* (comparative cycle threshold method [$2^{-\Delta Ct}$]). (*G*) GDF15 plasma levels analyzed by ELISA in serum of LD mice with leptin treatment (n = 7 to 11). (*H*) Relative mRNA expression of *GDF15* in liver tissue of LD mice treated with leptin (n = 3/group) by qPCR, norm. to *m36b4* mRNA ($2^{-\Delta Ct}$). (*I*) Spearman correlation of GDF15 and leptin level in plasma of control (n = 6), LD (n = 5), and LD + leptin (n = 6) mice.

Table 2. Characteristics of the LD study population (n = 102)

	Control participants*	LD*	$P^{\dagger,\ddagger}$
No.	54	48	
Age (y)	38.5 (22)	43.0 (26)	0.658
Sex (male/female)	12/42	11/37	
BMI (kg/m^2)	24.8 (4.9)	25.1 (4.0)	0.480
WHR	0.81 (0.13)	0.97 (0.11)	**<0.001**
SBP (mm Hg)	123.5 (22)	132.5 (22)	**<0.001**
DBP (mm Hg)	78 (12)	83 (16)	0.141
HbA_{1c} (%)	5.2 (0.6)	6.2 (2.3)	**<0.001**
HbA_{1c} (mmol/mol)	33.0 (6.4)	44.5 (25.2)	**<0.001**
FG (mmol/L)	5.3 (0.8)	6.2 (4.4)	**<0.001**
FI (pmol/L)	52.4 (45.6)	113.5 (126.3)	**<0.001**
HOMA-IR	1.75 (1.60)	4.95 (7.30)	**<0.001**
Cholesterol (mmol/L)	5.41 (1.38)	5.11 (2.10)	0.191
HDL cholesterol (mmol/L)	1.52 (0.58)	0.81 (0.48)	**<0.001**
LDL cholesterol (mmol/L)	3.59 (1.41)	2.28 (1.73)	**<0.001**
TG (mmol/L)	0.99 (0.61)	3.29 (6.67)	**<0.001**
FFA (mmol/L)	0.45 (0.23)	0.61 (0.28)	**0.018**
CRP (mg/L)	0.8 (1.8)	1.8 (2.9)	**0.006**
Adiponectin (mg/L)	9.2 (7.7)	2.5 (3.2)	**<0.001**
Leptin (μg/L)	12.4 (14.1)	3.9 (5.1)	**<0.001**
GDF15	414.9 (226.3)	830.6 (885.9)	**<0.001**

BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; FFA, free fatty acid; FG, fasting glucose; FI, fasting insulin; GDF15, growth differentiation factor 15; HbA_{1c}, glycosylated hemoglobin A_{1c}; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LD, lipodystrophy; LDL, low-density lipoprotein; SBP, systolic blood pressure; TG, triglyceride; WHR, waist to hip ratio.
*Data are reported as median (interquartile range).
†Mann-Whitney *U* test.
‡*P* values in bold type are statistically significant at <0.05.

After demonstrating a connection of leptin and GDF15 in ECs, the LD mouse model, and patients, we wanted to investigate a direct relation of GDF15 with atherosclerotic parameters in humans. Thus, we analyzed the association of GDF15 with the presence of carotid plaques, carotid intima-media thickness, ankle-brachial index, brachial-ankle pulse-wave velocity, and NT-proBNP using appropriate regression models adjusting for age, sex, body mass index, low-density lipoprotein cholesterol, diabetes status, smoking status (current smoker vs. nonsmoker), estimated glomerular filtration rate, and hypertension. *GDF15* was positively associated with brachial-ankle pulse-wave velocity ($\beta = 0.094$; $P = 3.1 \times 10^{-4}$), NTproBNP ($\beta = 0.103$; $P = 7.3 \times 10^{-4}$), and carotid plaque presence ($\beta = 0.394$; $P = 9.9 \times 10^{-4}$). Together, our data reveal that *GDF15* is associated with atherosclerosis in humans and is increased in EndMT and LD, which can be inhibited by additional administration of leptin.

Gene Expression After Leptin Treatment in Atherosclerotic Plaques of the Murine Lipodystrophy model. To investigate the effect of leptin on the vessel wall in vivo, we analyzed gene expression in atherosclerotic plaques in the murine LD model and after treatment with leptin by spatial transcriptomics. Brachiocephalic artery sections of wild-type, control ($Ldlr^{-/-}$), LD mice, and LD mice treated with leptin were used (Fig. 5*A*). Plaques were automatically identified by clustering (cluster 3) (Fig. 5*B*). Key elements of TGF-β signaling (*Tgfb1*, *Tgfb2*, and *Tgfbr1*), direct TGF-β targets (*Serpine1* and *Thbs1*), wound myofibroblast (*Lyz2*), and macrophage markers (*Gpnmb* and *Cd68*), as well as *Gdf15* in LD mice were increased and reduced after leptin treatment (Fig. 5 *C* and *D*). These data provide mechanistic insight on the effects of leptin on gene expression in atherosclerotic plaques in vivo reducing LD-induced, EndMT-related gene expression.

Inhibition of *GDF15* Reduces EndMT Induction. Due to the observed increased expression of *GDF15* during EndMT, we further characterized the role of *GDF15* during EndMT. Stimulation of primary human ECs with GDF15 induced EndMT, as indicated by increased expression of mesenchymal markers *SM22* and *CNN1* (Fig. 6 *A* and *B*). Functionally, GDF15 treatment reduced EC resistance—a sign of impaired endothelial barrier function (Fig. 6*C*). GDF15 is part of the TGF-β superfamily and promotes epithelial to mesenchymal transition; thus, we hypothesized that GDF15 action is mediated via TGFBR2 as the most highly expressed TGF-β receptor in ECs (*SI Appendix*, Fig. S2). Indeed, the *GDF15*-mediated effect on EndMT was mediated via *TGFBR2* signaling (Fig. 6 *D* and *E*). Furthermore, treatment of ECs with neutralizing antibodies or a small interfering RNA targeting *GDF15* reduced the EndMT induction in ECs (Fig. 6 *F–H*). Interestingly, addition of serum of patients with LD to ECs induced EndMT. The dependence of EndMT induction on GDF15 in sera from patients with LD was confirmed by additional treatment of ECs with neutralizing antibodies against GDF15, which reduced EndMT induction (Fig. 6 *I* and *J*). Finally, we found GDF15 serum concentrations in patients with LD to be reduced by leptin treatment for a period of 4 wk, as determined by the Olink assay and GDF15 ELISA (Fig. 6 *K* and *L* and Table 3). These data suggest that *GDF15* can induce EndMT and impair endothelial functions and, therefore, might be a new target in the therapy of LD (Fig. 6*M*).

Discussion

LDs are characterized by reduced adipose tissue mass and dysregulated adipokine serum concentrations. Leptin replacement therapy in patients with LD is a promising therapeutic approach, leading to improved glycemic control and decreased triglyceride levels and mortality (21, 22). LDs are associated with elevated risk of cardiovascular disease, and the precise causal mutation determines an individual's risk to CVD development (23). The important finding of the present study is that leptin treatment exerts direct effects on ECs by reducing inflammation, EC permeability, and EndMT. In generalized, aP2-nSrebp1c transgenic LD mice on a low-density lipoprotein receptor knockout ($Ldlr^{-/-}$) background, leptin replacement therapy reduced the number of ECs undergoing EndMT in vascular plaques, the accumulation of macrophages in atherosclerotic lesions, and the protrusion of atherosclerotic plaques. Mechanistically, we found evidence that the observed vasculopathy in LD is mediated by *GDF15*, which shows association with atherosclerosis in humans (24).

The lack of leptin in LD directly impairs the physiological functions of the vasculature, especially of ECs (25, 26). In this regard, leptin replacement therapy restores impaired endothelium-dependent relaxation in patients with LD, suggesting a direct influence of leptin on the endothelial layer (27). This is consistent with our observations of a direct effect of leptin on EC function in the context of atherosclerosis, such as improved endothelial barrier function (Fig. 1*J*).

We found a strong increase in EndMT in a 16-wk-old LD mouse model. This indicates the existence of a chronic disease mechanism underlying the increase in EndMT in LD. We were

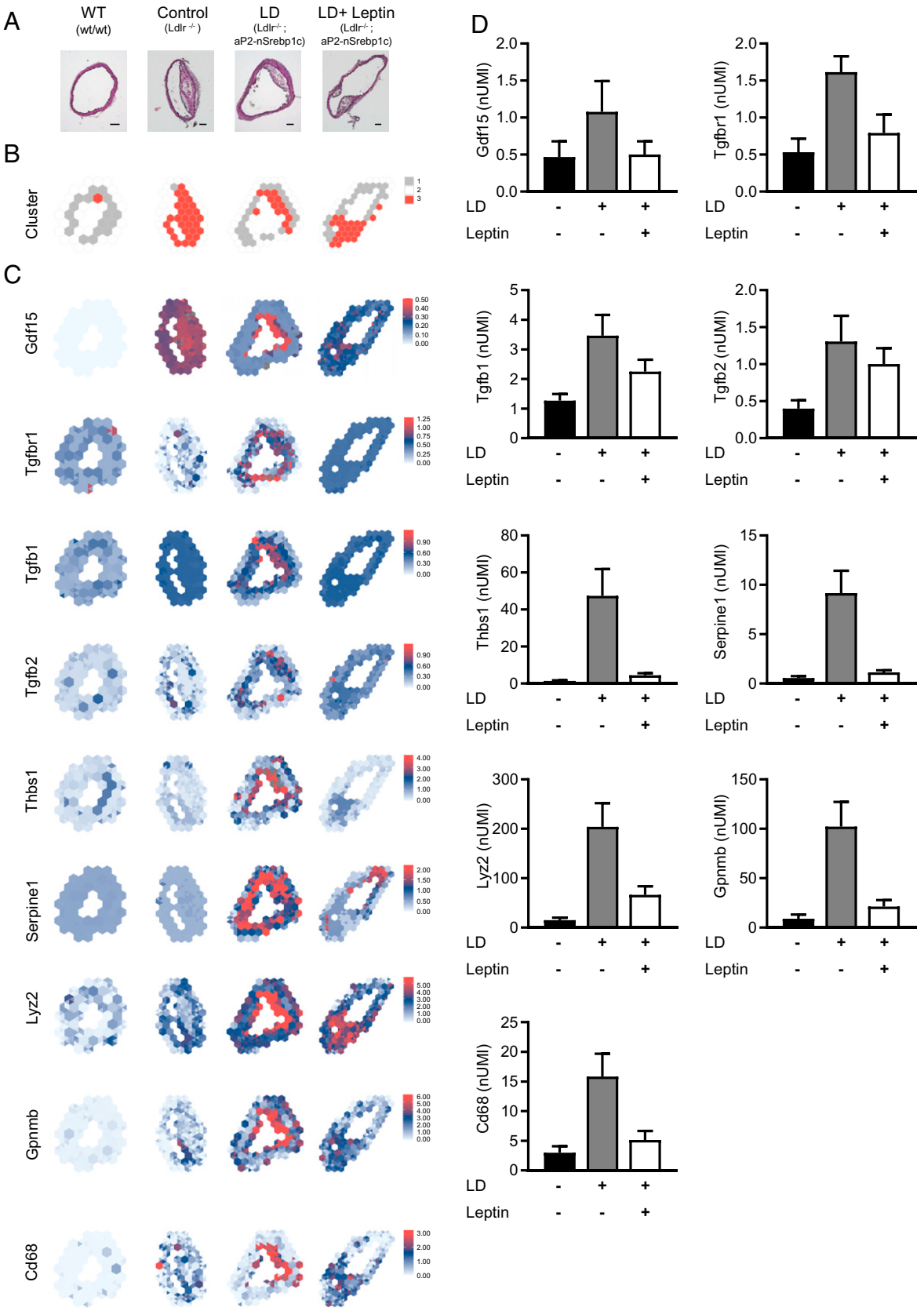

Fig. 5. Gene expression in atherosclerotic plaques of murine lipodystrophy model. (*A*) Brachiocephalic artery sections of wild-type (WT), control ($Ldlr^{-/-}$), LD mice, and LD mice treated with leptin. (*B*) Gene expression data in spots of WT (n = 45), control ($Ldlr^{-/-}$; n = 57), LD mice (n = 71), and LD mice treated with leptin (n = 76). Plaques were automatically identified by clustering (cluster 3). (*C* and *D*) Spatial gene expression (*C*) and quantification (*D*) as normalized unique molecular identifiers (nUMI) in spots of cluster 3 of selected genes in control mice ($Ldlr^{-/-}$; n = 30), LD mice (n = 13), and LD mice treated with leptin (n = 24), reported as mean + SEM.

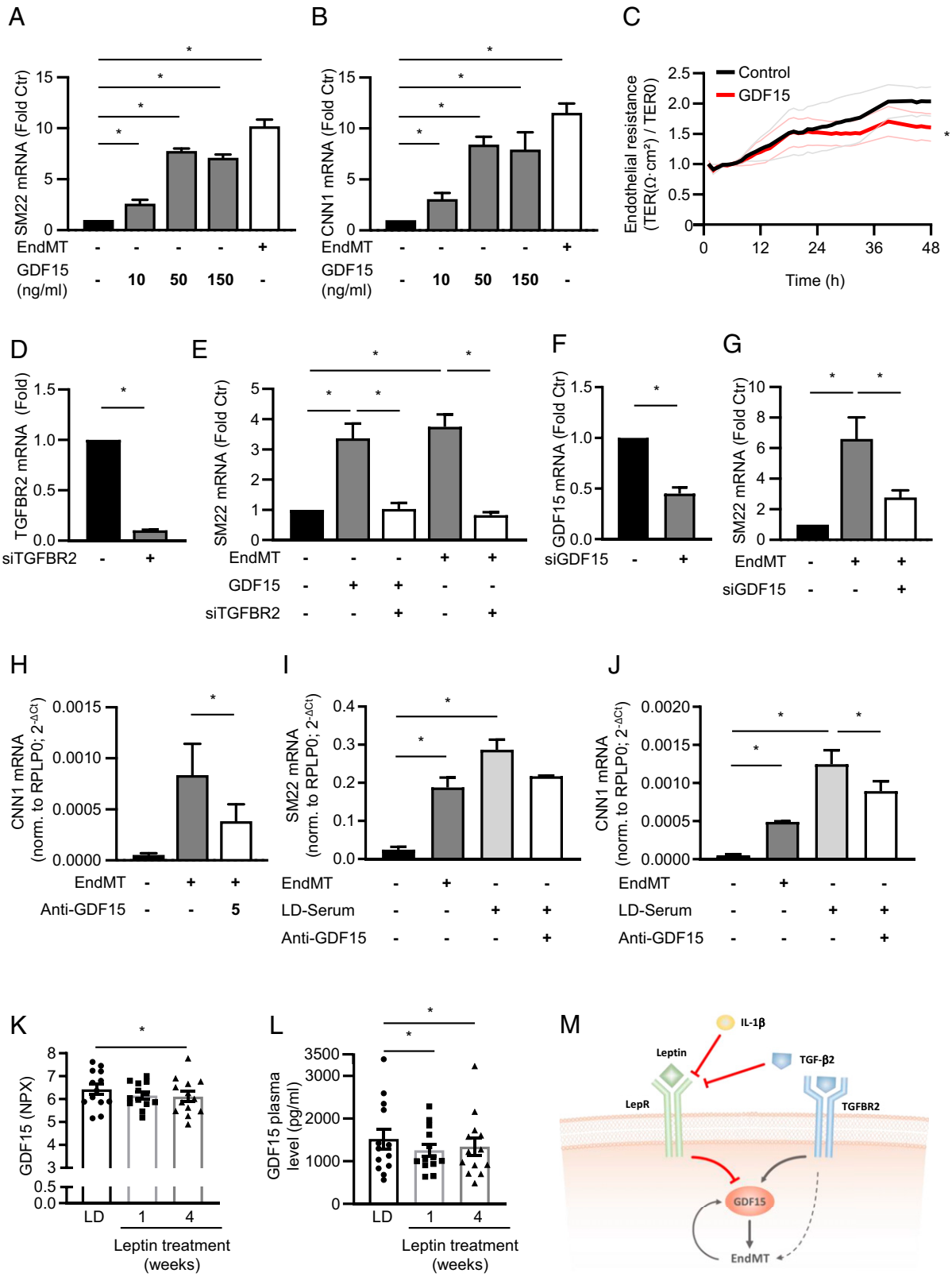

Fig. 6. *GDF15* induces EndMT-mediated impairment of ECs. (*A* and *B*) GDF15 effect on *SM22* (*A*) and *CNN1* expression (*B*) ($n = 3$), determined by qPCR, normalized (norm.) to *RPLP0* (comparative cycle threshold method [$2^{-\Delta Ct}$]). (*C*) Continuous impedance measurement of GDF15-treated ECs over a period of 48 h ($n = 3$). (*D* and *E*) *TGFBR2* ($n = 3$) (*D*) and *SM22* mRNA (*E*) expression after small interfering RNA targeting *TGFBR2* (si*TGFBR2*) with GDF15 treatment in ECs ($n = 3$), by qPCR norm. to *RPLP0* ($2^{-\Delta Ct}$). (*F* and *G*) *GDF15* (*F*) and *SM22* (*G*) mRNA after small interfering *GDF15* (si*GDF15*) in ECs ($n = 3$ to 5), determined by qPCR, norm. to *RPLP0* ($2^{-\Delta Ct}$). (*H*) *CNN1* after EndMT with GDF15-neutralizing antibodies ($n = 3$) by qPCR, norm. to *RPLP0* ($2^{-\Delta Ct}$). (*I* and *J*) *SM22* (*I*) and *CNN1* (*J*) after stimulation of ECs with serum of patients with LD with GDF15-neutralizing antibodies ($n = 3$), norm. to *RPLP0* ($2^{-\Delta Ct}$). (*K*) GDF15 protein measured with Olink assay in serum of patients with LD and after treatment for 1 and 4 wk of recombinant leptin ($n = 13$; normalized protein expression [NPX] ± SEM). *$P < 0.05$. (*L*) GDF15 protein measured with ELISA in serum of patient with LD and after treatment for 1 and 4 wk of recombinant leptin ($n = 13$). Data are shown as mean + SEM. *$P < 0.05$. (*M*) Schematic illustration of the proposed mechanism of *GDF15* and leptin in EndMT.

Table 3. Characteristics at baseline as well as at 1 wk and 4 wk after initiation of metreleptin treatment in patients with LD ($n = 16$)

	Before treatment*	At 1 wk*	$P^{\dagger,\ddagger}$	At 4 wk*	$P^{\dagger,\ddagger}$
No. of patients		16			
Age (y)		42 (18)			
Sex (male/female)		3/13			
BMI (kg/m^2)	27.4 (5.6)	27.4 (6.4)	0.082	27.2 (6.5)	0.197
WHR	0.97 (0.1)	0.98 (0.12)	1.000	0.95 (0.1)	0.288
SBP (mm Hg)	128 (16)	130 (29)	0.750	131 (13)	0.469
DBP (mm Hg)	80 (16)	80 (18)	0.532	77 (10)	0.691
HbA1c (%)	8.0 (2)	7.6 (1.8)	0.217	7.2 (1.3)	**0.003**
HbA1c (mmol/mol)	63.9 (23.8)	59.0 (19.7)	0.348	55.2 (14.0)	**0.002**
FG (mmol/L)	9.6 (2.9)	8.0 (4.5)	0.197	8.0 (3.6)	0.622
FI (pmol/L)	144.2 (280)	135.8 (337.4)	0.597	277.7 (417)	0.497
HOMA-IR	12.4 (11)	11.4 (14.1)	0.323	14.2 (21.6)	0.455
Cholesterol (mmol/L)	5.85 (5)	5.43 (2.39)	**0.033**	4.71 (2)	**0.008**
HDL cholesterol (mmol/L)	0.62 (0.48)	0.77 (0.58)	0.488	0.69 (0.7)	0.297
LDL cholesterol (mmol/L)	1.65 (2.09)	2.27 (2.27)	0.147	1.99 (1.75)	0.569
TG (mmol/L)	8.64 (14.8)	5.39 (5.21)	**0.004**	5.30 (5.4)	**0.003**
FFA (mmol/L)	0.70 (0.3)	0.61 (0.32)	0.197	0.51 (0.4)	0.191
CRP (mg/L)	3.1 (5.4)	3.5 (4.2)	0.808	4.3 (4.7)	0.392
Adiponectin (mg/L)	2.2 (1.8)	1.9 (1.5)	0.659	2.0 (1.6)	0.672
Leptin (μg/L)	5.1 (4.6)	7.5 (8.4)	0.072	6.8 (16)	0.340

BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FFA, free fatty acid; FG, fasting glucose; FI, fasting insulin; HbA_{1c}, glycosylated hemoglobin A_{1c}; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LD, lipodystrophy; LDL, low-density lipoprotein; SBP, systolic blood pressure; TG, triglyceride; WHR, waist to hip ratio.

*Data are reported as median (interquartile range) or absolute numbers.

†Wilcoxon signed-rank test.

‡*P* values in bold type are statistically significant at <0.05.

able to demonstrate that leptin treatment reduces EndMT in vitro and in vivo. During EndMT, ECs lose their prototypical endothelial barrier function (14).Thus, immune cells can infiltrate the vascular barrier more easily. In vitro, leptin treatment restored EC functions and preserved *CDH5* expression and organization, resulting in decreased endothelial permeability. In accordance, the in vivo LD model showed more macrophages infiltrating atherosclerotic lesions in the presence of EndMT, which was reversed by leptin treatment (Fig. 3*C*).

The LD mouse model we used is characterized by overexpression of sterol regulatory element-binding protein-1c (*SREBP-1c*), resulting in a phenotype comparable to human LD, making it a suitable model for analysis of this disease in mice (18). We generated a mouse model by combining the original aP2-nSrebp1c-Tg line with an $Ldlr^{-/-}$ mouse for the analysis of atherosclerosis. Our $Ldlr^{-/-}$; aP2-nSrebp1c-Tg line shows clear LD phenotypic changes, such as an increase in liver weight and a decrease in white subcutaneous and visceral fat content. In addition, our line responds to treatment with leptin, as shown by the improvement of hepatic markers ALAT and ASAT as well as a decrease in liver weight.

Leptin treatment in $Ldlr^{-/-}$; aP2-nSrebp1c-Tg mice reduced the number of cells undergoing EndMT and reduced the LD-mediated accumulation of macrophage in vascular lesions. The diminishing effects of leptin on EndMT were profound, whereas leptin did not affect the overall atherosclerotic plaque area. Leptin treatment exerted positive effects by reducing macrophage infiltration and plaque protrusion. These findings are important given that EndMT is a marker of plaque instability (16). The observations on plaque characteristics may reflect clinically relevant vasculo-protective effects. Macrophage infiltration is a hallmark of vulnerable atherosclerotic plaques that are prone to rupture and the underlying mechanism of atherothrombotic events such as myocardial infarction (28). Patients with protruding atherosclerotic plaques have a higher risk for embolic events, such as stroke (19, 29–31). Our findings are further supported by antiinflammatory effects and decreased atherosclerotic lesions in leptin-deficient $Ldlr^{-/-}$;*ob/ob* mice (32, 33) and the higher risk for vascular events seen in patients with lipodystrophy (23).

In contrast, an increase in total plaque burden after treatment of *ApoE*-deficient mice with leptin was shown before (34). However, the animals were not an LD model or atherosclerosis ($Ldlr^{-/-}$) model, as in our study. Furthermore, the animals were treated with a higher dose (125 μg/d) of leptin than in our study (3.0 mg/kg body weight per day). Indeed, high doses of leptin can exert a paradox of action, which, contrary to the benefits of low doses, leads to a deterioration in cardiovascular health at high doses (9). Here, we found treatment with 200 ng/mL leptin inhibited EndMT in ECs. Leptin exerts both dichotomous and paradoxical effects associated with cardiovascular function. Indeed, hyperleptinemia is positively correlated with unfavorable outcomes (9). In this regard, in vitro leptin concentrations at 50 to 250 ng/mL had a positive effect on cell proliferation and angiogenic properties (35), whereas concentrations high as 500 ng/mL were shown to be less favorable for ECs by inducing endothelin (36). The role of leptin in the cardiovascular system might be even more complex, given the previous reports in atherosclerotic mouse models (34, 37, 38).

GDF15 is a member of the TGF-β family (39). In the context of various diseases [e.g., cancer (40), cardiovascular disease (41, 42), obesity and type 2 diabetes (43)], an up-regulation in circulating *GDF15* has been observed. Furthermore, it was recently shown that in patients with LD and in lipodystrophic mice, serum concentrations of *GDF15* are elevated and could be down-regulated by leptin treatment (44). *GDF15* was shown to increase atherosclerotic plaque size, increasing the vulnerability of plaques and impairing ECs in plaques (24, 45, 46). On the basis of our data, we conclude that *GDF15* induces EndMT and that this atherosclerotic effect can be limited by leptin administration, which may be relevant to obesity in which *GDF15* is elevated (20).

Leptin acts through the leptin receptor (*LepR*), which is highly expressed by hepatocytes and ECs. We found *LepR* highly expressed in physiological ECs but markedly reduced under conditions of inflammation and EndMT (*SI Appendix*, Fig. S3). Indeed, increased levels of inflammatory cytokines were previously reported in the lipodystrophic mouse model we used for our study (47). That is a potential explanation for the lack of effect on total plaque area, where we also found the *LepR* reduced. Regulation of the *LepR*, therefore, might be a potential mechanism of leptin signaling in the vasculature. Interestingly, a beneficial inverse association between carotid artery wall thickness and circulating leptin levels was reported in healthy young individuals, thereby supporting a potential vascular protective role of leptin (48). However, such associations were not reported in obese patients with metabolic disorders and older individuals with overweight (49–52).

The findings of our study may help us understand the current paradox regarding the so-called acquired leptin resistance, in which leptin levels are high but have no functional effects (53, 54).

Our data in the context with the literature, especially in EC-specific, LepR knockout studies, strongly suggest that leptin replacement therapy in patients with LD not only exerts beneficial metabolic effects but may also directly improve vascular function (25). Loss-of-function mutation in genes resulting in congenital lipodystrophy in humans, such as *AGPAT2*, *BSCL2*, *CAV1*, or *PTRF*, were not regulated in our model of EndMT. Therefore, they are not likely to impact the effects of leptin on EndMT.

Based on our results, leptin is a factor controlling EndMT. EndMT is reversible and therefore represents a very attractive target for novel therapeutic strategies to inhibit atherosclerotic plaque progression and instability (55). The mechanisms described in the present study support the vasculo-protective role of leptin in LD and identify therapeutic approaches for the treatment of this disease that warrant testing in clinical studies.

Materials and Methods

Additional details about the methods we used in this study are provided in *SI Appendix, Materials and Methods*.

Generation of the *Ldlr*$^{-/-}$; aP2-nSrebp1c-Tg Mice. The LD-strain aP2-n*Srebp1c*-Tg was on a mixed C57BL/6 × SJL F2 background. For analysis of atherosclerosis in lipodystrophy, we needed to combine this line with the *Ldlr*$^{-/-}$ model on a C57BL/6 background. Therefore, we backcrossed the aP2-nSrebp1c-Tg mice to a clear C57BL/6 background over eight generations, verified by a 1,449 single nucleotide polymorphism marker panel (Taconic Bioscience). In contrast to the initial aP2-nSrebp1c-Tg mice, our generated *Ldlr*$^{-/-}$; aP2-nSrebp1c-Tg animals showed no signs of hyperglycemia (18).

Animal Care and Animal Experiments. Mouse breeding and experiments were approved by the local ethics committee (approval no. TVV37/12, 06/11/2012 and TVV 27/16, 22/09/2016). More details are provided in the *SI Appendix*, *Extended Methods*.

Statistics. Data are depicted as mean + SEM. Statistical significance was determined using Student's *t* test or ANOVA with post hoc analysis. A value of $P < 0.05$ was considered statistically significant.

Data, Materials, and Software Availability. Data deposition: The single-cell RNA sequencing data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE213438) (56).

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