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Sophie Reggio[1,2,3*], Christine Rouault [1,2,3 *], Christine Poitou[1,2,3], Jean-Christophe Bichet[4], Edi Prifti[1], Jean-Luc Bouillot[5], Salwa Rizkalla[1], Danièle Lacasa[2,3]*, Joan Tordjman[1,2,3]* and Karine Clément[1,2,3]*,

1 Institute of Cardiometabolism and Nutrition, ICAN, Assistance Publique-Hôpitaux de Paris, Nutrition department, Pitié-Salpêtrière hospital, F-75013, Paris, France;

2 INSERM, UMR_S U1166, Nutriomics team, F-75013, Paris, France;

3 Sorbonne Universités, UPMC Univ Paris 06, UMR_S 1166, F-75005, Paris, France;

4 Assistance Publique-Hôpitaux de Paris, Plastic surgery and Mammary Cancer department Pitié-Salpêtrière hospital, F-75013 Paris, France;

5 Assistance Publique-Hôpitaux de Paris, Visceral surgery department, Ambroise Paré Hospital, F- 92104, Boulogne, France

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* shared co-authorship

Corresponding author: Joan Tordjman

e-mail address: joan.tordjman@upmc.fr

Phone: 00 33 01 40 77 96 70 Fax: 00 33 01 40 77 97 04

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Abstract

Context: Collagen accumulation around adipocytes and vessels (i.e. pericellular fibrosis) is a hallmark of obese adipose tissue associated with altered metabolism.

Objective: To evaluate components of basement membrane (BM) in adipose tissue, including collagen IV, a major BM component, and its relationships with metabolic parameters and TGFβ isoforms.

Design and setting: We used immuno-techniques and gene expression approaches to detect BM components in subcutaneous and visceral adipose tissue samples. Adipocytes and endothelial cells were isolated from lean and obese adipose tissue. We also focused on the expression of *COL4A1* correlated to metabolic variables in moderate obesity and, in severe obesity before and after bariatric surgery. Using *in vitro* analysis, we explored the impact of TGFβ isoforms on the expression of inflammatory and extracellular matrix genes in adipocytes and endothelial cells.

Results: BM components were detected around adipocytes and endothelial cells, and were increased in obese adipocytes. *COL4A1* expression was positively correlated with insulin-resistance indices in obese subjects, and showed less reduction in severely obese subjects with poorer insulin-resistance outcomes six months after gastric bypass. *COL4A1* expression also correlated with *TGFβ1* and *TGFβ3* gene expressions in subcutaneous adipose tissue. Stimulating isolated adipocytes and endothelial cells *in vitro* with these TGFβ isoforms showed an inflammatory and pro-fibrotic phenotype. However, TGFβ1 and TGFβ3 exposure only provoked *COL4A1* over-expression in endothelial cells, and not in adipocytes.

Conclusion: The disorganization of several BM components, including collagen IV, **could contribute** to pathological alterations of obese adipose tissue and cells.

Introduction

Obesity and comorbidities are associated with major alterations of white Adipose Tissue (AT) (1). These alterations include increased interstitial fibrosis defined as an excessive accumulation of extracellular matrix (ECM) components (2). Over the last few years, relationships between fibrotic accumulation in AT and obesity-related metabolic deterioration, such as insulin-resistance and type 2 diabetes, have been described (3). At the level of AT, cell types such as adipocytes, progenitors, endothelial, and immune cells, are all embedded in a three-dimensional fibrillar network of ECM proteins (4,5). In human AT, we have observed increased expression (6) and deposition of ECM proteins, that surround not only adipocytes (i.e. called pericellular fibrosis) but also blood vessels (7). Several studies have also explored specific fibrillar collagens and collagen VI, whose expression in AT is associated with metabolic alterations in humans and rodents, and is regulated by weight loss (5,7,8).

A key component of the ECM is the basement membrane (BM), which is found in close proximity to other cell membranes. The BM of endothelial cells (EC) has been well described in many tissues (9), however in AT, these structures have only been demonstrated via morphological studies focusing on adipocytes (10). The BM provides cellular architectural support and also interacts with integrins for signaling (11). This interaction is crucial for correct cell behavior through outside-in signaling. BMs composition and supramolecular organization depend both on tissue type and specific developmental periods or pathophysiological events within those tissues. These modifications are well described, during glomerulogenesis for example, or diabetic nephropathy and retinopathy (12,13). Collagen IV and laminins are two major components, which self-assemble in the extracellular space to form a network which creates BM ultrastructure. Nidogen and perlecan (HSPG2) bridge the laminin and collagen IV network to stabilize and maintain BM integrity (9). The matricellular protein SPARC, expressed in AT (8), also contributes to BM stabilization.

Collagen IV, a heterotrimeric glycoprotein, accounts for 50% of the BM and is composed of up to six distinct alpha-chains, α1(IV) to α6(IV) (14). The chains interact and assemble with remarkable specificity due to specific recognition of non-collagenous domains (7S and NC1) to form three distinct heterotrimers: α1α1α2, α3α4α5 and α5α5α6 (15). *COL4A1* and *COL4A2* are the most common isoforms and are present in all tissues. Laminins consist of assembled α, β and γ chains, and five α, three β and three types of γ chains have been identified in vertebrates (9). Depending on the chain combination, at least 15 laminin isoforms have been described (9). Laminin knockout mouse models have highlighted the importance of certain chains in a functional ECM structure (16). For example, γ1 null mice are embryonically lethal, producing disorganized extracellular deposits of collagen IV and perlecan (17), showing that this ubiquitous subunit is key for BM assembly. Thus structural anomalies of BM can lead to tissue dysfunction and eventual lethality in some models (18). Among these anomalies, BM thickening is commonly associated with tissue dysfunction, as shown in diabetic nephropathy or retinopathy (12,13). Collagen IV overexpression and oversecretion in the extracellular space is a hallmark of BM thickening (19), and the pro-fibrotic factor, TGFβ1, is an important molecular contributor (20,21).

Today, morphological analysis has highlighted the presence of BM around adipocytes in AT (10), but no study has specifically explored BM components in lean and obese AT cells, nor the potential relationships between the major BM component collagen IV and metabolic alterations in obesity. Moreover, whereas previous studies showed that TGFβ1 is expressed in AT and is associated with BMI (22), no study has evaluated the potential relationships between TGFβ isoforms, collagen IV, and ECM components in obesity.

Thus, using a combination of immuno-technique microscopy and gene expression approaches in human AT samples, we examined BM composition and its variations in adipocytes from both lean and obese subjects. We also assessed the relationships between collagen IV expression and metabolic phenotypes in obese subjects. Finally using 3D *in vitro* models, we

explored the effect of TGFβ isoform on the BM, and pro-fibrotic or pro-inflammatory genes in adipocytes and EC from AT.

Materials and Methods

The list of antibodies and chemical compounds used in this study are presented in the Supplemental Data.

Subjects

First, we obtained **subcutaneous periumbilical** AT (SAT) samples by needle aspiration from an initial cohort of 60 obese subjects (F/M: 40/20, mean age: 51.29 ± 1.18 and BMI: 31.37 ± 0.44 kg/m^2) as described in (23). These subjects were treatment naïve and had impaired fasting glucose. Subjects were without overt diabetes, and had normal renal, hepatic and thyroid function. We obtained serum glucose and insulin after 12-hours of fasting and calculated insulin resistance indexes from HOMA-IR and HOMA-%S measurements, and beta cell function HOMA-%B using the Homeostatic Model Assessment Insulin Resistance (24).

Secondly, 25 severely obese women candidates for bypass surgery (mean age: 48.9 ± 1.8 and BMI: 47 ± 1.4 kg/m^2) were selected. Among them, 16 subjects were non-diabetic and 9 had type 2 diabetes. The subjects had not been dieting before the surgery and their weight had been stable for at least three months prior to surgery. Subcutaneous periumbilical adipose tissue (SAT) samples were obtained by needle aspiration before the surgical procedure (T0). This group of patients was followed-up six months after the bariatric surgery and we collected SAT **at the periumbilical location using the same procedure (i.e. that needle biopsy (T6))**. Patients were characterized by a series of bioclinical factors just prior to, and six months following surgery, as previously described in (25). **For microscopy analysis, paired SAT (periumbilical) and VAT (omental) sample biopsies were obtained from 5 morbidly**

obese subjects (mean age: 38.5 ± 11.6, BMI: 44.7 ± 7.5 kg/m^2, F/M: 4/1) during surgery by the same surgeon (JLB). A portion of each sample was fixed in 4% formaldehyde for immunohistochemistry/immunofluorescence analysis and 0.2% glutaraldehyde-2% paraformaldehyde for immuno-electron microscopy analysis.

Thirdly, AT biopsies were also obtained from non-obese women (mean age: 46.5 ± 5.1 and BMI: 23 ± 0.5 kg/m^2). These subjects had been admitted for scheduled abdominal surgery. **Subjects were exempt from acute or chronic inflammatory or infectious disease, viral infection, cancer and/or known alcohol consumption** and consented to AT sampling from **periumbilical location** as obese subjects.

All participants gave written and informed consent. All clinical investigations were performed according to the Declaration of Helsinki and approved by the Hôtel-Dieu hospital ethics committees.

Microscopic Analysis of human AT

Immunofluorescence and immunohistochemistry analyses were performed on serial sections of AT from five lean and five obese subjects as described in (26) and (27) respectively. Negative controls were performed by omitting primary antibodies. Immuno-electron microscopy was performed in human SAT as described in supplementary methods.

Isolation of adipocytes and endothelial cells from human SAT

SAT biopsies were digested with collagenase then adipocytes and EC were isolated as described in (28) and (29) respectively. For each cells types, one part was kept for gene expression analysis and the other for culture experiments.

3D cultures of isolated adipocytes

Adipocytes were cultured in a 3D-cultured model as described in (26). Culture medium was changed every other day.

Gene expression studies

Cultured adipocytes and EC remained untreated or were treated with 5 ng/ml TGFβ1 or TGFβ3 for 48h. mRNA expression was determined by the Quantigene Plex 2.0 Reagent System (QuantiGene Affymetrix, CA, USA) as described in supplementary methods.

Gene expression was quantified by RT-qPCR in adipocytes or EC isolated from human AT. RNA extraction, reverse transcription, and real-time PCR were conducted as described previously (29). Primers are listed in Supplemental Table 1. Values were normalized to *18S* expression.

Statistical Analysis

Data were analyzed using GraphPad software (San Diego, CA). Values are expressed as means ± S.E.M. Differences between **gene expressions** were determined with non-parametric Mann-Whitney tests (obese *vs*. lean subjects) or Wilcoxon tests (SAT *vs.* VAT; prior to *vs.* six months after surgery **and in vitro studies**). **Differences between clinical data (prior to *vs* six months after surgery) were determined using paired non-parametric Mann-Whitney tests.** Correlations were examined with non-parametric Spearman correlations. Results were considered significant when $p < 0.05$.

Results

Basement membrane components in human lean adipocytes and endothelial cells

To investigate the BM in AT, we first performed immunofluorescence microscopy to detect collagen IV protein in SAT from non-obese subjects. This initial morphological exploration revealed collagen IV staining surrounding adipocytes and blood vessels (Figure 1A). Immuno-electron microscopy confirmed that collagen IV staining was located proximate to adipocytes (Figure 1C and **Figure S1A-S1D**) and EC membranes (data not shown). In isolated adipocytes and EC we assessed the expression of genes encoding several BM components, including *COL4A1*, a ubiquitously expressed α chain and *LAMC1*, the most commonly expressed laminin chain. We also evaluated the gene expression level of other BM components such as *NID-1* and *HSPG2*, as well as *SPARC*, a matricellular protein involved in BM stabilization.

COL4A1 was expressed at similar levels in isolated adipocytes and in EC (Figure 1E). In contrast, *COL4A3* or *COL4A5* transcripts were not detected (data not shown). Expression levels of *LAMC1*, *NID-1*, and *HSPG2*, were similar in lean adipocytes and EC, whereas *SPARC* had increased expression in isolated adipocytes compared to EC ($p=0.018$) (Figure 1E). These observations demonstrate that similar to EC, isolated adipocytes express several key BM components, including *COL4A1*.

Basement membrane components increase in obese AT

Via immunofluorescent studies, collagen IV staining was more intense around adipocytes and blood vessels in obese compared to lean SAT samples (Figure 1A-B).

Immuno-electron microscopy analysis confirmed not only the increased amount of collagen IV close to adipocyte membranes, but also different patterns in obese samples. Collagen IV appeared more disorganized with frequent associated clusters around obese compared to lean adipocytes (Figure 1D). We also found an AT depot-dependent pattern of collagen IV.

Collagen IV staining was much more intense in SAT from both lean and obese subjects, compared to VAT from both lean and obese subjects (Figure 1G-J).

BM gene expression profiles correlated with histological findings. *SPARC* gene over-expression was observed in obese adipocytes (p=0.021), which also demonstrated increased *COL4A1* (p=0.001), *LAMC1* (p=0.003), *NID-1* (p=0.041) and *HSPG2* (p=0.05) gene expression compared to that of lean adipocytes (Figure 1F). The expression levels of *COL4A1*, *NID-1* and *SPARC* were also significantly higher in adipocytes isolated from obese SAT compared to VAT (*COL4A1*: p=0.005; *NID-1*: p=0.026; *SPARC*: p=0.002) (Figure 1K). *COL4A1* expression was significantly correlated with *LAMC1*, *NID-1*, and *SPARC* genes in both SAT and VAT (Table S2). Both morphological and gene expression explorations demonstrated increased BM components in obese adipocytes, suggesting they could contribute to BM thickening.

Collagen IV gene expression in obese SAT is associated with glucose metabolism

In diseases such as diabetic nephropathy, increased collagen IV is induced by hyperglycemia. We thus explored potential links between *COL4A1* expression in SAT (SAT-*COL4A1*) and glucose metabolism from i) moderately obese subjects with increased fasting blood glucose and, ii) severely obese subjects before and after gastric bypass, a condition known to drastically improve glucose metabolism.

In 60 obese subjects with impaired fasting glucose but no anti-diabetic treatment (Table S3), we observed significant correlations between SAT-*COL4A1* expression and markers of glucose homeostasis, such as fasting glucose and insulin, markers of insulin resistance (HOMA-IR and HOMA-%S) and of β-cell function, HOMA-%B, as shown in Table 1. These associations remained after adjusting for weight (data not shown).

We next investigated the relationship between variations in SAT-*COL4A1* expression and glucose metabolism improvement in 16 non-diabetic subjects with severe obesity before and

after gastric bypass (Table S4). Six months after bariatric surgery, SAT-*COL4A1* expression decreased by 29.5% ($p<0.01$) (Fig. 2A). Individual responses to bariatric surgery intervention were highly variable and treatment dependent, consequently we used a clustering approach to stratify the 16 non-diabetic subjects into two equivalent partitions using the median value for each clinical and biological parameter. For each clinical variable, we were then able to identify those that improved the most (top half) and those with little or no improvement (bottom half). Using these partitions, we found that obese subjects with the greatest improvement in HOMA-IR, the insulin-resistance surrogate, were those in which SAT-*COL4A1* decreased the most following surgery ($p=0.014$) (Figure 2B). No additional relationships were detected between variations in SAT-*COL4A1* following surgery, and other biological or clinical improvement parameters. We also observed that the other previously described BM components were significantly decreased following surgery (**Figure S2A-S2C**). Using the same clustering approach for *COL4A1*, we determined that SAT-*NID1* was also related to HOMA-IR improvement (data not shown).

Collagen IV gene expression in obese SAT is associated with TGFβ isoforms

TGFβ1, a major pro-fibrotic growth factor correlated with BM thickness in several tissues (30). However this specific aspect has never been explored in obese AT. Moreover, using previous microarray data from our laboratory, we have observed that another TGFβ isoform, *TGFβ3*, was also highly expressed in human AT while *TGFβ2* was barely detected (data not shown). We then explored the relationship between both *TGFβ1* and *TGFβ3* and BM components in obese SAT.

Similar to *COL4A1*, the expression of both SAT-*TGFβ1* and SAT-*TGFβ3* decreased by 23% and 20% respectively six months after bariatric surgery (Figure 2C and 2D). We found strong positive correlations between SAT-*COL4A1* and SAT-*TGFβ1* expression, as well as between SAT-*COL4A1* and SAT-*TGFβ3*, in the group sampled prior to surgery (Figure 2E and 2F).

These associations remained at six months following the weight loss induced by bariatric surgery (data not shown). We also found positive associations between variations in SAT-*COL4A1* expression and variations in both SAT-*TGFβ1* (Figure 2G) and SAT-*TGFβ3* (Figure 2H) between baseline and six months post-surgery. Of note, the other previously explored BM components were also positively correlated to SAT-*TGFβ1* or SAT-*TGFβ3* expression. To a lesser extent, we also found significant positive associations between the variations in *LAMC1*, *HSPG2*, and *SPARC* expression and those of *TGFβ1* and *TGFβ3* (**Figure S2D**). These associations suggest potential relationships between pro-fibrotic TGFβ isoforms and the modulation of BM components, particularly *COL4A1*.

TGFβ1 and TGFβ3 effects on human adipocytes and endothelial cells

Since SAT-*TGFβ1* and SAT-*TGFβ3* were associated with upregulated SAT-*COL4A1* expression (and other BM components), we hypothesized that these pro-fibrotic factors could induce the synthesis of BM components in AT cells. TGFβ1 is indeed known to induce *COL4A1* over-expression in epithelial cells (31). We used a previously described 3D model to test the effects of these TGFβ isoforms on the genetic expression of two BM components (*COL4A1*, *LAMC1*) and a series of ECM remodeling markers (*COL3A1*, *COL6A3*, *SPARC*, and *LOX*, as well as *CTGF*, *TGFβ1*, *PAI-1*, and *α-SMA*) and inflammation markers (*CCL2*, *IL-6*), in isolated human lean adipocytes and EC.

Firstly, we observed that TGFβ1 treatment induced significant over-expression of *PAI-1*, *TGFβ1*, *CTGF* and *IL6* in both 3D cultured adipocytes (Figure 3A) and EC (Figure 3C). TGFβ1 treatment also increased *SPARC* and *LOX* expression in both types of isolated cells.

In contrast, the gene expression of BM components (*COL4A1*, *LAMC1*) and other collagens (*COL3A1*) in isolated adipocytes was not significantly changed by TGFβ1 stimulation (Figure 3B). *COL4A1* and *COL6A3* expression were however significantly induced by TGFβ1 in EC

(Figure 3D). As observed for TGFβ1, TGFβ3 treatment also provoked a significant up-regulation of *PAI-1*, *TGFβ1*, *α-SMA* and *IL6* in both 3D cultured adipocytes (Figure 4A) and EC (Figure 4C). *CTGF* expression was also strongly induced under these conditions (Figure 4C).

Importantly, TGFβ3 treatment only induced expression of *COL4A1*, *COL6A3*, and *LOX* in EC (Figure 4D).

Discussion

Here we have provided novel insights regarding the expression of BM components in adipocytes and EC, and their modifications in AT during human obesity. We have shown that adipocytes from obese AT samples exhibit augmentations in several BM components. Amongst them *COL4A1* expression positively associates with markers of **insulin resistance**, and with two TGFβ isoforms in obesity. *In vitro* data on human cells indicated that while TGFβ1 and TGFβ3 can stimulate remodeling and the up-regulation of pro-fibrotic and inflammatory genes in both adipocytes and EC, they can only induce *COL4A1* expression in EC, and not in isolated human adipocytes.

The BM of EC has been characterized in many tissues, but rarely in human AT, and has never been compared to isolated adipocytes. We observed that EC and adipocytes express common BM components such as a collagen IV protomer (α1α1α2), a laminin heterotrimer (containing the γ1 chain), nidogen and perlecan. While also detected in both of these cell types, transcripts of the matricellular protein SPARC were more highly expressed in adipocytes. This glycoprotein exhibits diverse functions, such as modulating the interaction of cells with the ECM (both the BM and the interstitial matrix) and BM permeability.

By focusing our study on a few major BM components, we can theorize that while adipocytes and EC share some common structural components, organizational differences could be

highlighted by variable *SPARC* gene expression, which as others modulates BM architecture to adapt to cell functions.

Obesity induces significant BM component modifications in both EC and adipocytes, as illustrated by increased deposition of collagen IV protein around these cells, and increased BM gene expression in obese compared to lean adipocytes. The increase in BM components was also greater in SAT compared to VAT. Our team recently highlighted the importance of collagen accumulation located not only around adipocytes but also EC (i.e. called pericellular fibrosis) in obese AT (7). Here it is tempting to consider that increases in BM components around these cells, such as collagen IV, contribute to peri-adipocyte and peri-vascular fibrosis. Additional detailed imagery analysis of human AT would be necessary to provide further insights regarding the structural organization of pericellular fibrosis.

In many tissues such as kidney or retina, BM remodeling is related to insulin resistance and a hyperglycemic environment. We have previously shown that pericellular fibrosis in AT is associated with deterioration of metabolic parameters, and also with diabetes in severe obesity (3,32). BM components were not explored in this pathological context, instead focus was made on collagen VI, a type of collagen, which forms the interface between BM and thick bundles of collagen I. The expression of collagen VI in AT has been correlated to glucose metabolism impairment in human populations (33). Here, we have shown that the expression of collagen IV, a major component of adipose BM, also associates with several variables related to glucose homeostasis and insulin-resistance in obese individuals with impaired fasting glucose and naïve of treatment. While this quantitative association was not observed at baseline in more severe forms of obesity, *COL4A1* expression in AT significantly decreased six months after bariatric surgery, and was associated with improved metabolic condition. This observation correlates with a previous study from our team demonstrating that the gene expression of several types of collagen were down-regulated in AT one year after gastric bypass (32). In the current study, subjects with the greatest HOMA-IR improvement had the

lowest *COL4A1* expression six months after the surgery. These data are only correlative, but they do help predict the impact of hyperglycemic/hyperinsulinemic tissue environments on BM component synthesis in AT cells.

Deeper insights into molecular interactions are also needed. As such, TGFβ isoforms are relevant candidates as in addition to their pro-fibrotic role, TGFβ1 can induce collagen IV expression and BM thickening in other tissues, such as kidney and retina. This link may also exist in AT. Our association studies indeed showed strong positive correlations between *COL4A1* and *TGFβ1* expression in the SAT of severely obese subjects, both prior to, and six months post-bariatric surgery. Similar associations were found for the TGFβ3 isoform, also highly expressed in AT. These observations prompted us to examine whether both TGFβ isoforms could induce the expression of *COL4A1* and other remodeling genes in human isolated adipocytes and EC, using a previously described *in vitro* 3D model (26). Both TGFβ1 and TGFβ3 induced the expression of several pro-fibrotic and inflammatory genes in adipocytes and EC, but *COL4A1* expression was only significantly induced in EC.

This raises important questions about whether other pro-fibrotic stimuli can induce adipocyte BM remodeling and change ECM composition in obese AT. In our 3D model, we tested other known collagen IV-inducers, such as activin A, but neither induced BM component expression in isolated adipocytes (data not shown).

Recently, one study demonstrated that another TGFβ superfamily member, BMP4, induces *COL4A1* and *COL4A2* expression in isolated glomeruli from mouse kidneys (34). While a recent study highlighted that BMP4 has an anti-inflammatory effect in adipocytes, its action on collagen IV expression in adipocytes should also be explored (35).

While obesity is characterized by deregulated production of adipokines, it remains also to understand whether the two main adipokines, leptin and adiponectin, could also contribute to fibrosis development in AT. Indeed in liver, leptin exerts pro-fibrotic impact while adiponectin is anti-fibrotic (36). In a recent in vitro study performed on

HUVEC, leptin treatment induced collagen IV over-secretion while when adding adiponectin, collagen IV secretion returns to control secretion (37). Based on these findings, it would be interesting to assess further the effects of these adipokines on the secretion of collagen IV in endothelial cells and adipocytes. Overall further studies are needed to understand how the production of basement membrane components by adipocytes is regulated.

Treating isolated adipocytes and EC with TGFβ isoforms induced *LOX* expression, an enzyme that plays a key role in catalyzing covalent collagen crosslinks in the extracellular environment, and regulates ECM mechanical properties. Both adipocytes and EC may thus contribute to AT fibrosis via the thickening of their own BM due to pro-fibrotic stimuli. We have also shown that *LOX* expression is induced in obese AT (28) and is decreased after weight loss (32) suggesting modulation of collagen cross-linking with subsequent effects impacts on cell constraints. **Furthermore, AT tensile strength has been shown to be a feature that promotes co-morbidities development (38) suggesting a potential role of BM component in this phenomenon.**

Our laboratory indeed demonstrated that adipocytes could also be considered as mechano-sensitive cells subject to bilateral mechanical forces during obesity (28). On one hand, lipid droplet growth exerts physical stress from inside the cell, and on the other, AT fibrosis characterized by fibrillar cross-linked collagen can create constraints from the outside in. Interestingly, collagen IV knockout studies in *C. elegans* and mice have demonstrated that this BM component is crucial in enabling cells to respond to mechanical constraints by transmitting signals between cells and interstitial matrix (39,40). It is thus tempting to suggest that collagen IV accumulation around obese adipocytes could be governed by mechanical stresses, either induced by lipid droplet enlargement and/or fibrosis accumulation. Further studies are needed to investigate this aspect in more depth.

In conclusion we have described major BM component modification in adipocytes and EC during obesity. Collagen IV is a major BM component associated with *in vivo* metabolic alterations in human obesity, thus future molecular studies are needed to determine both its regulation and contribution to altered adipocyte biology.

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Figure legends

Figure 1: **BM components in lean and obese human adipose tissue and isolated adipocyte and endothelial cells.**

(A-D) Collagen IV immunofluorescence in lean **(n=5)** (A) and obese **(n=5)** human subcutaneous adipose tissue collected by surgical biopsy (B) Collagen IV immuno-electron microscopy in lean (C) and obese (D) human subcutaneous adipose tissue. Representative samples are shown from a set of five explored samples. (E) *COL4A1*, *LAMC1*, *NID-1*, *HSPG2* (perlecan), and *SPARC* expression was quantified by real-time PCR and normalized to *18S* in adipocytes (n=12; white bar) and compared to endothelial cells (n=5; grey bar) from lean subcutaneous adipose tissue. (F)- *COL4A1*, *LAMC1*, *NID-1*, *HSPG2* (perlecan), and *SPARC* expression was quantified using real-time PCR and normalized to *18S* in lean (n=12; white bar) or obese (n=12; black bar) adipocytes. (G-J) Collagen IV immunohistochemistry in either lean **(n=5)** human subcutaneous, (G) or visceral adipose tissue (I); and in obese **(n=5)** human subcutaneous (H) or visceral adipose tissue (J). Representative samples are shown after the histological analysis of 5 subjects for each condition. (K) *COL4A1*, *LAMC1*, *NID-1*, *HSPG2* (perlecan), and *SPARC* expression was quantified by real-time PCR and normalized to *18S* in adipocytes isolated from either obese subcutaneous (n=16; black bars) or visceral adipose tissue (n=16; hatched bars). **For RT-qPCR, values are obtained from the average of the reference sample Ct.** Ad: adipocytes; EC: endothelial cells; SAT: subcutaneous adipose tissue; VAT: visceral adipose tissue; arrow: collagen IV. Data are expressed as mean ± SEM. Significant differences between groups are indicated *** $p<0.001$; **$p<0.01$; *$p<0.05$ Mann-Whitney's test or Wilcoxon's test for subcutaneous versus visceral adipose tissue comparison.

Figure 2: Decreased expression of collagen IV, TGFβ1, and TGFβ3 genes in subcutaneous adipose tissue following weight-loss is associated with improved glucose metabolism parameters.

(A). *COL4A1*, (C) *TGFβ1*, and (D) *TGFβ3* gene expression was quantified by real-time PCR and normalized to *18S* in human obese subcutaneous adipose tissue before (black bars) and after weight loss (hatched bars). T0: Before weight loss; T6: six months after weight loss; SAT: subcutaneous adipose tissue. (B). Average *COL4A1* expression in subcutaneous adipose tissue after patient samples were clustered depending on HOMA-IR improvement six months after surgery. Data are expressed as mean ± SEM. Statistically significant differences between groups are indicated ** $p<0.01$; *$p<0.05$. n=25. Correlation analysis between subcutaneous adipose tissue *COL4A1* gene expression and *TGFβ1* before (E) and six months after surgery (G); and *TGFβ3* before (F) and six months after surgery (H). Wilcoxon's tests were used in (A), (B), (C), and (D), and Pearson's correlations were used in (E), (F), (G), and (H). n=25

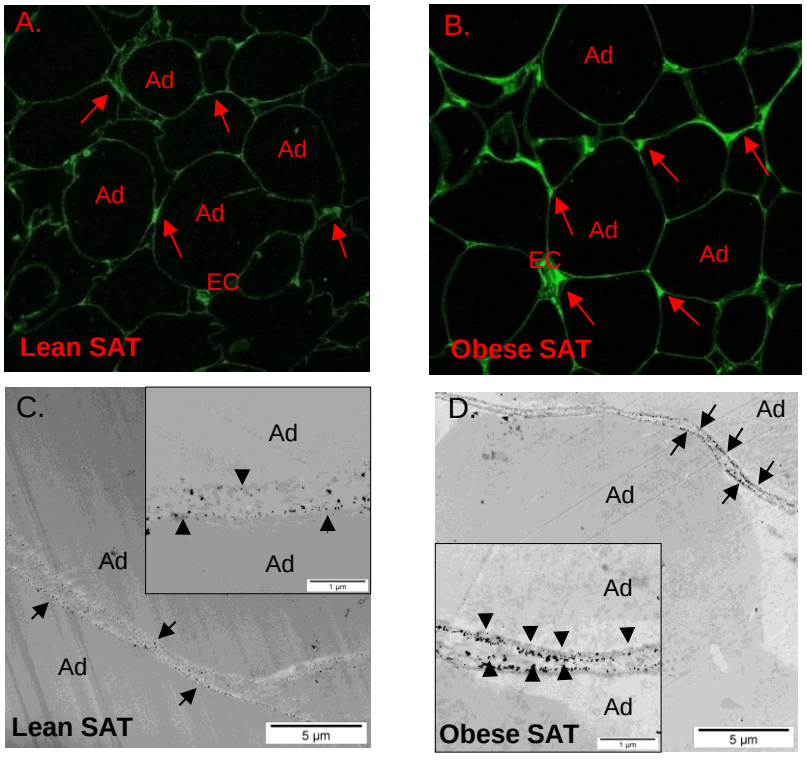
Figure 3: TGFβ1 effects on ECM and inflammatory genes in 3D adipocytes and endothelial cells isolated from lean human subcutaneous adipose tissue.

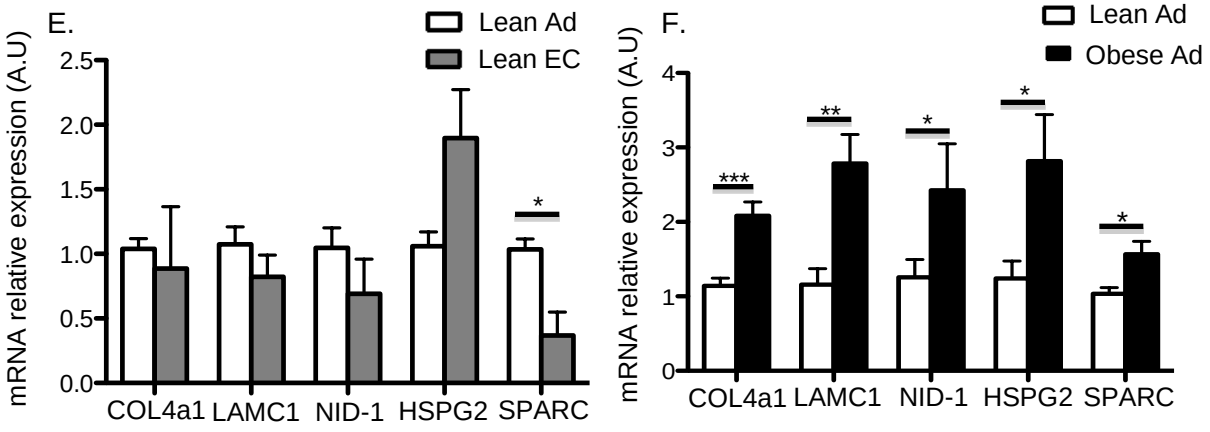
3D adipocytes and endothelial cells were treated for 48h with 5 ng/mL recombinant TGFβ1. (A-B) Inflammatory genes were quantified by Quantiplex Assays in either non-treated (white bars) or treated (black bars) 3D adipocytes (A); and non-treated (light grey bars) or treated (dark grey bars) endothelial cells (B). (C-D) ECM remodeling genes were quantified by Quantiplex Assays in either non-treated (white bars) or treated (black bars) 3D adipocytes (C); and non-treated (light grey bars) or treated (dark grey bars) endothelial cells (D). Ad: adipocyte (n=12); EC: endothelial cells (n=8). Statistically significant differences between groups are indicated *** $p<0.001$; ** $p<0.01$; *$p<0.05$ Wilcoxon's test.

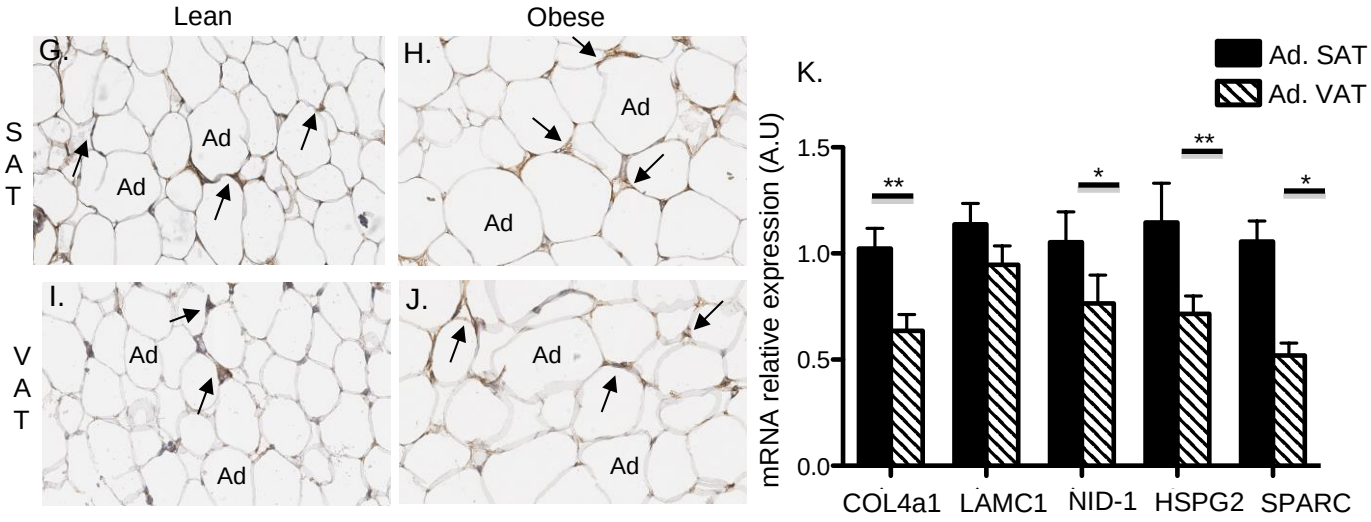
Figure 4: TGFβ3 effects on ECM and inflammatory genes in 3D adipocytes and endothelial cells isolated from lean human subcutaneous adipose tissue.

3D adipocytes and endothelial cells were treated for 48h with 5 ng/mL recombinant TGFβ3. A-B Inflammatory genes were quantified by Quantiplex Assays in either non-treated (white bars) or treated (black bars) 3D adipocytes (A); and non-treated (light grey bars) or treated (dark grey bars) endothelial cells (B). C-D ECM remodeling genes were quantified by Quantiplex Assays in either non-treated (white bars) or treated (black bars) 3D adipocytes (C); and non-treated (light grey bars) or treated (dark grey bars) endothelial cells (D). Ad: adipocyte (n=9); EC: endothelial cells (n=6). Statistically significant differences between groups are indicated *** $p<0.001$; ** $p<0.01$; *$p<0.05$ Wilcoxon's test.

Figure 1


A.
Ad
Ad
Ad
Ad
Ad
EC
Lean SAT
B.
Ad
Ad
EC
Ad
Obese SAT
C.
Ad
Ad
Ad
Ad
Lean SAT
5 µm
D.
Ad
Ad
Ad
Ad
Obese SAT
5 µm


E.
Lean Ad
Lean EC
mRNA relative expression (A.U)
2.5
2.0
1.5
1.0
0.5
0.0
*
COL4a1 LAMC1 NID-1 HSPG2 SPARC
F.
Lean Ad
Obese Ad
mRNA relative expression (A.U)
4
3
2
1
0
***
**
*
*
*
COL4a1 LAMC1 NID-1 HSPG2 SPARC


Lean
Obese
SAT
VAT
G.
Ad
Ad
H.
Ad
Ad
I.
Ad
Ad
J.
Ad
Ad
K.
Ad. SAT
Ad. VAT
mRNA relative expression (A.U)
1.5
1.0
0.5
0.0
**
*
**
*
COL4a1 LAMC1 NID-1 HSPG2 SPARC

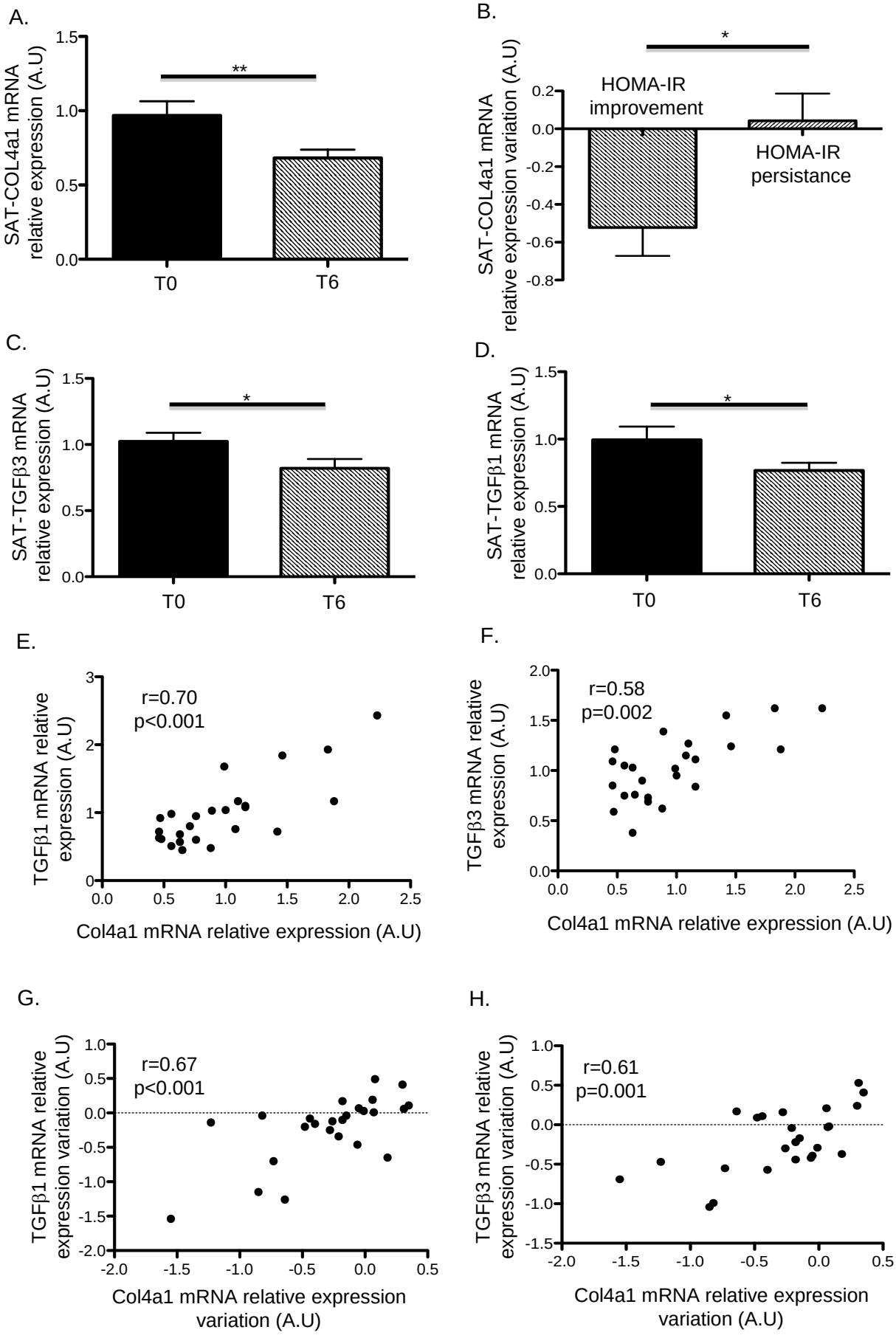

A.
SAT-COL4a1 mRNA relative expression (A.U)
1.5
1.0
0.5
0.0
**
T0
T6
B.
SAT-COL4a1 mRNA relative expression variation (A.U)
*
HOMA-IR improvement
HOMA-IR persistance
0.2
0.0
-0.2
-0.4
-0.6
-0.8
C.
SAT-TGFβ3 mRNA relative expression (A.U)
*
T0
T6
D.
SAT-TGFβ1 mRNA relative expression (A.U)
*
T0
T6
E.
TGFβ1 mRNA relative expression (A.U)
r=0.70
p<0.001
Col4a1 mRNA relative expression (A.U)
F.
TGFβ3 mRNA relative expression (A.U)
r=0.58
p=0.002
Col4a1 mRNA relative expression (A.U)
G.
TGFβ1 mRNA relative expression variation (A.U)
r=0.67
p<0.001
Col4a1 mRNA relative expression variation (A.U)
H.
TGFβ3 mRNA relative expression variation (A.U)
r=0.61
p=0.001
Col4a1 mRNA relative expression variation (A.U)

Figure 3

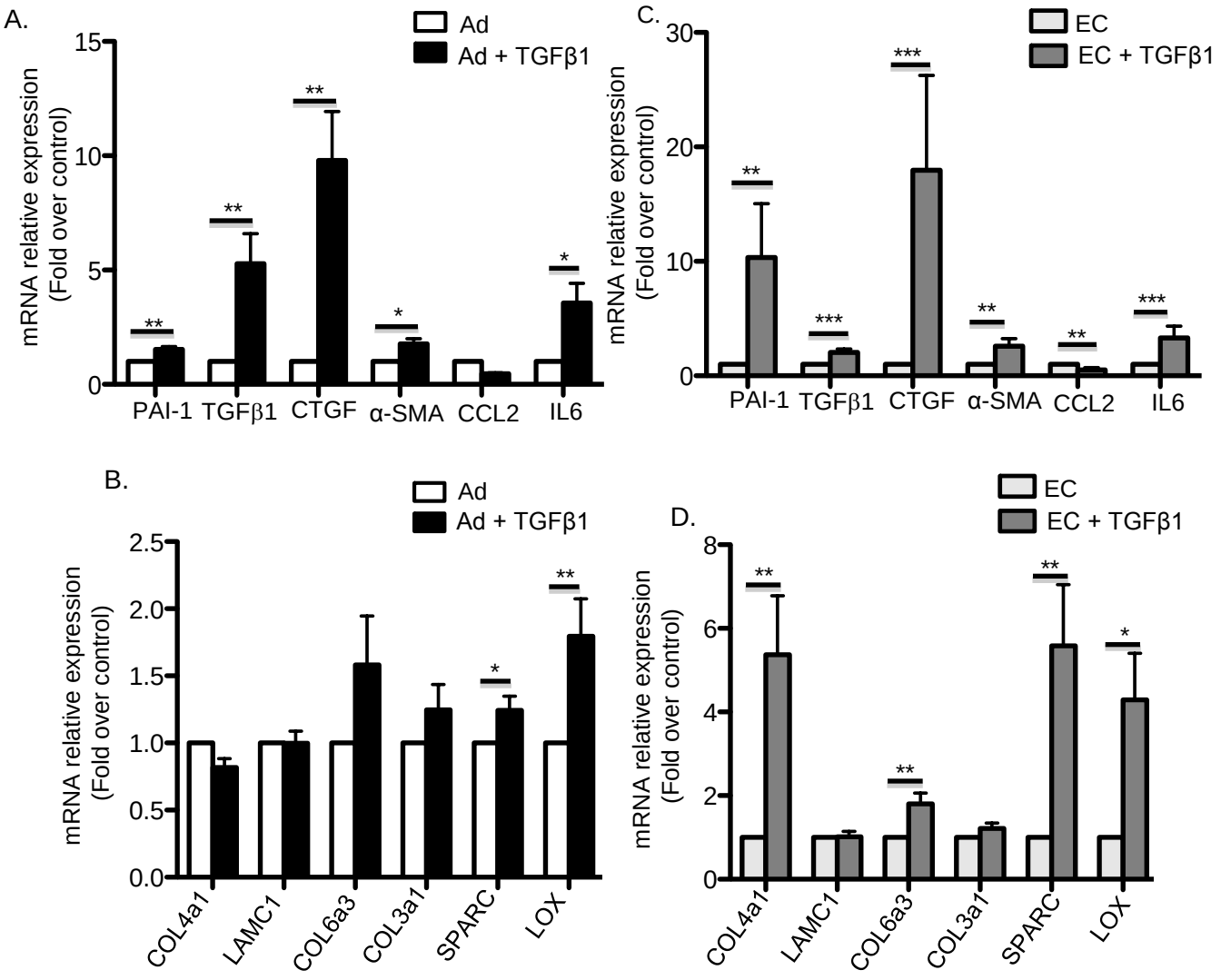
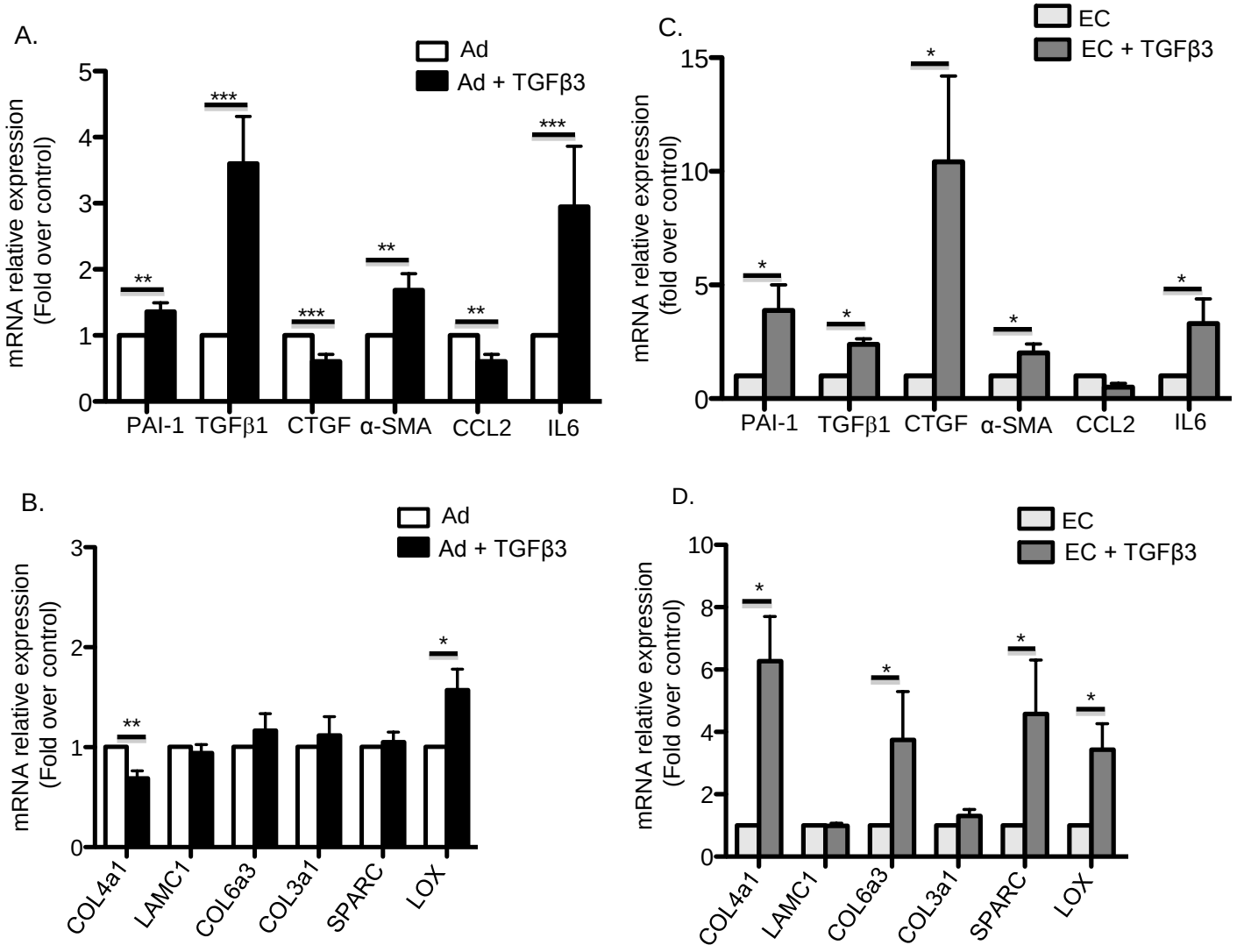

A.
Ad
Ad + TGFβ1
mRNA relative expression
(Fold over control)
15
10
5
0
**
**
**
*
*
PAI-1
TGFβ1
CTGF
α-SMA
CCL2
IL6
C.
EC
EC + TGFβ1
mRNA relative expression
(Fold over control)
30
20
10
0
**
***
***
**
**
***
PAI-1
TGFβ1
CTGF
α-SMA
CCL2
IL6
B.
Ad
Ad + TGFβ1
mRNA relative expression
(Fold over control)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
COL4a1
LAMC1
COL6a3
COL3a1
SPARC
LOX
D.
EC
EC + TGFβ1
mRNA relative expression
(Fold over control)
8
6
4
2
0
**
**
**
*
COL4a1
LAMC1
COL6a3
COL3a1
SPARC
LOX

Figure 4


A.
Ad
Ad + TGFβ3
mRNA relative expression
(Fold over control)
PAI-1
TGFβ1
CTGF
α-SMA
CCL2
IL6
B.
COL4a1
LAMC1
COL6a3
COL3a1
SPARC
LOX
C.
EC
EC + TGFβ3
D.