SUPPLEMENTAL MATERIAL

METHODS

KLF11-Luc promoter constructs

A 2kb KLF11 promoter region (-1910/+144) was amplified from genomic DNA using the GeneAmp PCR system (Applied Biosystems) with a forward primer (5’-AGAACCGCTCTGGTGAGACAGATGAT-3’) containing a MluI site and reverse primer (5’-TGCAAGCTTGCCGGAGCAACAAAG-3’) with a HindIII site. The amplified fragment was cloned into the pGL3-basic luciferase vector (Promega, Charbonnières, France). The PPRE mutation was introduced using the QuickChange site-directed mutagenesis kit (Stratagene) and the primers 5’-CGCCGCTGCGAAAATTCCCTTACTTTCCAG-3’ and 5’-CTGGAAAGTAAGGGAATTTTCGCAGCGGCG-3’. The sequences of all constructs were confirmed by automated sequencing (Applied Biosystems).

Transfection procedure

HMEC-1 cells were seeded at 40x10³ cells/well in a 24-wells plate and transfected with PromoFectin-HUVEC transfection reagent (Promocell GmbH). After 24 h, the cells were lysed in the reporter lysis buffer (Promega) and luciferase gene expression was monitored. β-galactosidase activity was measured as a control of the transfection. Each transfection experiment was done in triplicate and the results expressed as mean relative light units normalized to β-galactosidase activity. For siRNA studies, HMEC-1 cells were transfected with siRNA at a concentration of 50nM using the Dharmafect transfection reagent (Dharmacon, Thermo-Fisher Scientific) and RNA levels were measured by real-time quantitative PCR. ON-TARGETplus SMARTpool KLF11 and PPARα siRNAs from Dharmacon were used. SiRNA TCF7L2 (sense 5’-CCCACCCUCUUCAGAUGGAAGCUUA-3’; antisense 5’-UAAGCUUCCAUCUGAAGGGUGGG-3’) (TCF7L2HSS110547) were used.

RNA analysis

HMEC-1 cells were incubated with vehicle or FF (100μM) in MCDB131 medium containing 0.2% FCS in the presence or absence of TGFβ (10ng/ml), for the indicated times. HMEC-1 cells were incubated with vehicle, GSK3 inhibitor IX (50nM),
Kenpaullone (2.3µM), Wortmannin (1µM) or insulin (100nM) in MCDB131 medium containing 0.2% FCS, for the indicated times. RNA extraction was performed using TRIzol reagent (Invitrogen) and reverse transcription of 1µg RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time quantitative PCR analyses were performed using the Brilliant SYBR Green QPCR Master Mix on the Mx4000 detection system (Stratagene). The primers were human ET-1 5’-CCACCTGGACATCATTTGGGTCA-3’ and 5’-CCCTGAGTTCTTTTCTGCTTG-3’; human KLF11 5’-AGCATCTTGAGCAGACA-3’ and 5’-TGCACAGTGGTGGTGACA-3’; cyclophilin 5’-GCATACAGGTCCTGCTTCTTGTCC-3’ and 5’-ATGGTGATCTTCTTGGCTTGCTTG-3’

Samples were analyzed in triplicate in two independent runs. Ct values were determined for ET-1, KLF11 and normalized to the Ct of cyclophilin using the following equation: Relative values = 2^((Ct target gene−Ct cyclophilin)).

Chromatin immunoprecipitation assays

ChIP experiments were performed as described in1. Immunoprecipitation was performed with the goat polyclonal antibody anti-KLF11 (sc-23162) from Santa Cruz. Final DNA extractions were PCR-amplified using primer pairs that cover the three proximal KLF sites in the ET-1 promoter. The primers were 5’-AGGGAGAGCATTCCCTTGGT-3’ and 5’-GTCGGAGCTGTTTACCCCA-3’.

The PCR products were also analyzed by real-time quantitative PCR using the same primers pair.
Suppl. Figure I: PPARα expression is increased in confluent cells and this is correlated with an increase in KLF11 mRNA and protein expression. (A) PPARα- and KLF-11 mRNA expression was measured in non-confluent (NC) and confluent (C) cells using QPCR and normalized to the cyclophilin mRNA (mean value ± SEM of triplicate determinations). KLF11 protein expression was analyzed by western blotting using anti-KLF11 antibodies (B).
Suppl. Figure II: The pathway β-catenin and LEF/TCF is not involved in the FF-mediated reduction of ET-1 expression. It has been published that GSK3β down-regulation increases TCF7L2 expression\(^2\). Therefore, we tested the implication of this pathway in FF-mediated reduction of ET-1 expression. (A) HMEC-1 cells were transfected with TCF7L2- or ctrl-siRNA and cell lysates analyzed for ET-1 expression by QPCR and normalized to cyclophilin mRNA (mean value ± SEM of triplicate determinations). Statistical differences were calculated using unpaired t-test (one-tailed) (DMSO versus FF: p-value=0.0114 for ctrl siRNA; TGFβ versus TGFβ+FF: p-value=0.0221 for ctrl siRNA; DMSO versus FF: p-value=0.0084 for TCF7L2 siRNA; TGFβ versus TGFβ+FF: p-value=0.0280 for TCF7L2 siRNA; ctrl- versus TCF7L2-siRNA: p-value=0.0182 for DMSO). (B) HMEC cells transfected with TCF7L2- or ctrl-siRNA were analyzed by QPCR for TCF7L2 and normalized to the cyclophilin mRNA.
Suppl. FigureIII: The Smad proteins control ET-1 transcription via a mechanism involving GSK3. We have tested Smad as a potential target of this pathway because it has been published that GSK3β negatively regulates gene expression through interaction with Smad3. HMEC-1 cells were transfected with a specific Smad3 reporter plasmid (CAGA-luc) and 24h after transfection, treated with FF, GSK3 inhibitor IX (IX) or Kenpaullone (K) with or without TGFβ for 24h. The cell lysates were analyzed for the luciferase gene expression and β-galactosidase activity was measured as a control of the transfection. Each bar is the mean value ± SEM of triplicate determinations. Statistical differences were calculated using unpaired t-test (one-tailed) (TGFβ vs TGFβ+FF: p-value=0.0116; TGFβ vs TGFβ+IX: p-value=0.0112; TGFβ vs TGFβ+K: p-value=0.0111).
Suppl. Figure IV: FF decreases the binding of AP-1 on the ET-1 promoter in the presence of TGFβ probably contributing to the stronger effect of FF observed when cells are treated with TGFβ. Confluent HMEC-1 cells were treated with vehicle, FF, TGFβ or both compounds. ChIP assays were performed using anti-c-Jun antibodies (sc-44x) from Santa Cruz. Total extracts (input) were used as controls. The samples were analyzed by semi-QPCR using the oligonucleotides pair 5’-GGGCGTCTGCCTCTGAAGTTAGCAG-3’ and 5’-GACTTGGACAGCTCTCTGCC-3’ surrounding the AP-1 binding site located in the proximal part of the ET-1 promoter. The oligonucleotides pair 5’-GGTCAAAAGTTGGCCAAAAGGT-3’ and 5’-ACTGAGCCGGAAGCCAG-3’ located in the distal part of the ET-1 promoter was used as a negative control.
Suppl. Figure V: Full GSK3 activity is necessary for the ET-1 expression induction by TGFβ. Confluent HMEC-1 cells were treated with vehicle, TGFβ, TGFβ+GSK3-inhibitor IX (IX) or TGFβ+Kenpaullone (K). Cells were analyzed for ET-1 mRNA expression by QPCR and normalized to cyclophilin mRNA (mean value ± SEM of triplicate determinations). Statistical differences were calculated using unpaired t-test (one-tailed) (ctrl vs TGF: p-value=0.0385; TGFβ vs TGF+IX: p-value=0.0033; TGFβ vs TGFb+K: p-value=0.0083).
REFERENCES

