MATERIAL AND METHODS

Mouse adventitial progenitor cells isolation, sorting and culture. Mouse vascular progenitor cells were derived from the outgrowth of adventitial tissues as previously described[1] . AdvSca1 cells were selected with anti-Sca1 immunomagnetic microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany), sorted with a magnetic cell separator and cultured in stem cell culture medium composed of Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Rockville, Massachusetts, USA) with 10% ES Cell Qualified Fetal Bovine Serum (FBS) (Embryomax, Millipore), 10 ng/ml leukemia inhibitory factor (Merck Millipore LIF1050), 0.1 mM β-mercaptoethanol (GIBCO), 100U/ml penicillin/streptomycin (GIBCO) and 2mM L-glutamine (GIBCO). AdvSca1 cells were passaged every 3 days at a ratio of 1:3 on gelatin coated flasks. Cells were sorted for Sca1+ marker every 5 passages.

Transdifferentiation of AdvSca1 cells towards the endothelial lineage. AdvSca1 cells were plated in stem cell media without LIF. At day 2, the medium was replaced with a differentiation medium composed of KO DMEM (ThermoFisher) supplemented with 10% KO serum replacement (Life technologies) 0.1mM β-mercapto-ethanol, 100U/ml penicillin/streptomycin, 2mM L-glutamine and 25 ng/mL of recombinant murine VEGF165 (Peprotech). Adenovirus overexpressing mouse cDNA for ETV2 and adenovirus control (NULL) were purchased from ABM. Titer was evaluated using QuickTiter Adenovirus Titer ELISA Kit (Cambridge biosciences LTD). AdvSca1 cells were infected with a MOI of 100. Differentiation medium and VEGF were replaced at day 4 and 6. The cells were harvested at day 7.

Rat artery conditioned medium (CM). Ascending portions of rat aortas were isolated, flat opened and cut in two equal pieces. One part was used to injure *ex vivo* the endothelial layer using a cotton bud and the other one was left uninjured as described by Li et al[2]. Aorta fragments were then placed in 600 µl of ATCC DMEM at 37°C with antibiotics. Conditioned medium was collected after 48 h. 300 µL of DMEM medium (10% embryomax, without LIF) was then added with 300 µL of conditioned media from uninjured and injured aorta on 5000 AdvSca1 cells plated the day before. The media was replaced (300 µL DMEM+10% Embryomax+300 CM) after 3 days. Cells were fixed or harvested at day 5 for immunofluorescence or quantitative RT PCR analysis.

Microarray analysis. Mouse genome-wide expression analysis were performed using Affymetrix GeneChip Mouse Gene 2.0 ST Arrays. Total RNA from three lines of mouse AdvSca1, Adv_NULL, SMC, and 2 lines of Adv_ETV2 and EC (MS1 cells, ATCC CRL-2279) as well as one additional mouse EC line (yolk sak EC, ATCC, CRL-2581) were extracted using QIAGEN RNeasy Mini kit. Data were analyzed using Affymetrix expression console and transcriptome analysis console 3.0. Hierarchical clustering of global gene expression following microarray analysis was performed using a filter of -3.5/+3.5 for the differentially expressed genes between Adv_NULL and Adv_ETV2 groups. Gene ontology (GO) enrichment analysis was also performed using online software (**geneontology**.org) to compare upregulated and downregulated biological processes between respectively Adv_NULL/Adv_Sca1 cells and Adv_ETV2/Adv_NULL cells. Genes upregulated or downregulated more than 2 folds between the 2 groups according to the microarray data were used for the analysis.

AdvSca1 progenitor cells /SMCs co-culture and SMC proliferation assay. 30 000 SMCs were plated on cell culture inserts (Sls lifesciences, 3531102) in DMEM, 10% FBS on top of progenitor cells plated the day before in 6 wells plates (10^5 cells/well). 48 to 72 h later, SMC were incubated with Edu (10µM) for 2h, harvested, fixed and permeabilized. Edu was then detected using Click-it Plus Edu Flow Cytometry Assay kit (molecular probes, C10632), DNA content was stained using Fx cycle Far red Stain (Invitrogen, F10348). Cells were then

analyzed by flow cytometry and the results were analyzed using FlowJo software. Results presented are representative of two independent experiments performed in triplicates.

Transwell migration assay. On the lower chamber of transwell inserts with 8.0 µm pore size membrane filters, 800 µL of 0.2% FBS, ATCC medium was added. Next, $5x10^4$ cells of AdvSca1 cells previously cultured for 5 days in FBS+LIF or SR were resuspended in 200 µL of medium and were loaded into the upper chamber of the transwell insert. After 6 hours or overnight incubation, non-migrating cells on the upper chamber side of the filters were carefully washed with PBS and removed using a cotton bud. The migrated cells on the lower side of the membrane were fixed with 4% PFA in PBS for 10 minutes at room temperature and then stained with 0.1% crystal violet dye for at 15 minutes at room temperature. After a final washing step, the inserts were dried the migrated cells were counted in 5 different fields of each insert at 10X magnification. Data was expressed as the fold of migrated AdvSca1_SR compared to AdvSca1 progenitors in FBS+LIF (n=3, Student's T test was used for statistical analysis)

RFP labelling of the cells. Lentiviral particles were generated by transfecting HEK293T cells with LV H2b_RFP plasmid (addgene. #26001) and used to stably label the nucleus of adventitial cells. Efficiency of the infection was assessed by fluorescence microscopy. One flask of 175 cm^2 infected AdvSca1 RFP cells was passaged in 3, to keep AdvSca1 cells for 7 days or generate Adv_NULL and Adv_ETV2 cells. Consequently, cells from same batch, same passage, and same level of infection were used for the *in vivo* experiments (Matrigel plug assay and femoral artery wire injury).

Mice. All procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. C57BL/6J mice were purchased from Harlan, UK. Sca_GFP transgenic mice were purchased from The Jackson Laboratory (Bar Harbour, Maine, USA).

Aortic ring angiogenesis assay. Aortic rings were isolated from ApoE Sca1-GFP transgenic mice, placed in Matrigel, and cultured in OptiMEM (ThermoFisher) supplemented with 25ng/mL of recombinant mouse VEGF (Peprotech) for 7 days in the presence of control NULL or ETV2 overexpressing adenovirus. Medium and adenoviral particles were replaced every 2 days. The aortic rings were then fixed and processed for immunofluorescence analysis, using antibodies against cd31 (abcam, ab28364, 1/50) or ETV2 (Santa cruz, sc-164278, 1/50).

Femoral artery wire injury. Mice were anesthetized with ketamine and medetomidine hydrochloride and the surgical procedure was performed as previously described[3]. Femoral arteries were injured by insertion of a guide wire (CROSS-IT 100XT, HI-TORQUE). The adventitial side of the arteries were seeded with stably labelled H2b_RFP AdvSca1, Adv_NULL and Adv_ETV2 cells ($1x10^6$ cells) within 25 µl Matrigel® Basement Membrane Matrix (Corning). An injured artery with no seeded cells served as a control. Arteries were harvested 7 or 14 days following the injury and used to prepare frozen (for immunofluorescence) or paraffin sections (for Hematoxylin and Eosin, Elastica Van Giesen and immunohistochemistry). Measurements of the adventitia, media and neointima and calculation of the intima/media and adventitia/ media ratios were made on 3 hematoxylin and eosin stained sections per injured artery (n=6 arteries for each group).

***In vivo* Matrigel Plug Assay**. 10^6 stably H2b_RFP labelled AdvSca1 Adv_NULL or Adv_ETV2 were mixed with 50 µl of PBS and 250 µl Matrigel. The cell mixture was injected subcutaneously into the mice. Five injections were conducted for each group. The plugs were harvested 7 days later. Samples were fixed in liquid nitrogen, cryosections were prepared and immunofluorescence staining was then performed.

RT-PCR. Total RNA was isolated using a QIAGEN RNeasy Mini kit according to the manufacturer's instructions. 400ng to 1µg of RNA were reverse transcribed into cDNA with random primers using the MMLV reverse transcriptase kit (Promega).

Quantitative Real Time Polymerase Chain Reaction. Real time PCR was performed in technical duplicates using 20 ng of cDNA per sample with a SYBR Green Master Mix in a 20 µl reaction. Ct values were measured using the Eppendorf Mastercycler ep Realplex and GAPDH was used as an endogenous control to normalize the amounts of RNA in each sample. A list of the primers used for PCR amplification is provided at the end of this section.

Western Blot. Cells were lysed with lysis buffer in the presence of protease inhibitors (Santa Cruz), and protein concentrations were measured using the Bradford method.15 µg of protein lysate were applied to 4-12% Bis-Tris Protein gels (NuPAPE, Novex) before being transferred to a nitrocellulose membrane (Amersham Biosciences), followed by a standard western blotting procedure. The membrane was incubated with primary antibodies overnight at the following dilutions: VE-cadherin (abcam, ab33168, 1/300), αSMA (sigma, A5228, 1/1000), PDGFRβ (abcam, ab32570 1/2500), GAPDH (santa cruz, sc25778, 1/200). The next day, the membrane was incubated with appropriate secondary antibody at 1/10000 dilution (Dako) for 2 hours at room temperature before revelation.

Flow cytometry. Cells were harvested, fixed with 2% PFA for 20 min, washed with PBS and incubated with conjugated antibody for 30 mins on ice before flow cytometry. Compensation was performed and data was analyzed using FlowJo software. Conjugated antibodies list: anti-mouse Ly6A/E (Sca1) PerCP-Cyanine 5.5 PercP (eBiosciences, 45-5981), anti-mouse cd34 FITC (BD Pharmingen 553733), anti-mouse CD202b (Tie2) PE (eBiosciences, 12-5987), anti-mouse CD140b (PDGFRβ) PE (eBiosciences, 12-1402).

TET1/2/3 and IGFBP-5 gene knockdown. Tet1 (D-062861-01), Tet2 (D-058965-02), Tet3 (D-054156-01), IGFBP-5 (D-054834-01, D054834-02) siRNA and Non-Targeting siRNA control (D-001210-01-05) were purchased from Dharmacon (siGENOME). 60000 cells were plated on 6 well plates the day before. The next day, medium was replaced with DMEM with FBS or SR and siRNA was performed using Lipofectamine RNAiMAX transfection reagent (ThermoFisher) and following the manufacturer's instructions. Two siRNA targeting different sequences of the TET and IGFBP-5 genes were transfected. 72h after transfection, the level of down regulation was assessed using real time qPCR and the results for the most efficient siRNA (TET) or both siRNA (IGFBP-5) are represented. Experiments were performed in triplicates and are representative of 2 independent experiments.

Dot Blot. Genomic DNA was extracted using Wizard Genomic DNA purification kit (Promega) following manufacturer's instruction. For Dot Blot, genomic DNA of Adv_Sca1 cultured in FBS+LIF or SR for 5 days (3 independent experiments) was denaturated for 10 min at 95°C and 200 ng were placed on a nitrocellulose membrane (Amersham Biosciences) which was air dried and UV crosslinked for 15 min. The membrane was then blocked with 5% milk in TBST for 1h, and incubated overnight 4°C with 5hmc antibody (diagenode, pAb-HMC-050, rabbit polyclonal, 1/2500). Another membrane was blotted using a single stranded DNA antibody to control equal DNA amount (Millipore, MAB 3034 1/2500). The membrane was then incubated 1h with the secondary antibody (Dako, 1/10000) at room temperature before revelation. Quantification of 5hmc and ssDNA signals was performed using Image G software.

Acetylated low density lipoprotein (ac-LDL) uptake assay. Cells were incubated with 10µg/ml Alexa Fluor 594 conjugated Ac-LDL (ThermoFisher, L-35353) for 4 hours, stained with DAPI and pictures were taken under fluorescence microscope. Images are representative of 3 independent experiments.

Immunofluorescence Staining. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 10% swine serum for 1 hour at room temperature. Incubation with primary antibodies or Sma-Cy3 conjugated (sigma) was performed at 4℃ overnight, followed by incubation with secondary antibodies (corresponding fluorescent-conjugated IgG antibodies, ThermoFisher) for 45 mins at 37℃. Cells were then counterstained with DAPI for 3 mins at room temperature and mounted with fluorescent mounting media (Dako). For 5hmc staining, a denaturation step (2N HCl for 15 min), followed by a neutralization step (100mM Tris-HCl pH 8,5 for 10 min) were added before blocking. Antibodies: 5hmc (Diagenode, pAb-HMC-050, rabbit polyclonal, 1/500), VEGFR2 (Cell signalling, 2479S, 1/50), Tet1 (Novus, NBP219290, 1/100), Tet2 (Bioss, bs-9449R, 1/100), Tet3 (Novus biologicals, NBP2-20602, 1/100), ETV2 (Santa cruz, sc-164278, 1/200), β-catenin (Cell signalling, L87A12, 1/1000), Rasip1 (Proteintech, 17971-1, 1/200), IGFBP-5 (Santa cruz, sc-13093, 1/100), COL1A (Santa cruz, sc-8784, 1/50). Images are representatives of at least 3 independent experiments.

Hematoxylin and Eosin staining. Femoral arteries were fixed in 4% paraformaldehyde overnight. Later, the tissues were processed, embedded in paraffin, sectioned, and 5µM thick sections were processed for Hematoxylin and Eosin staining, Elastica Van Giesen staining or Immunohistochemistry.

Immunohistochemistry. Briefly, sections were processed through xylene and alcohol series followed by antigen retrieval in citrate buffer, treated with 3% H2O2 for 20 min, blocked in 10% serum for 30 mins followed by incubation with cd31 (abcam, ab28364, 1/50), or PCNA (abcam, ab29, 1/100) antibodies overnight at 4°C. Later, the sections were washed (PBS), incubated with biotinylated secondary antibodies (vectorlabs, 1/100) for 30 min at RT before revelation, and counterstaining with hematoxylin and mounting.

Statistics. Statistics were performed using Student's t-test or One-way ANOVA with Bonferroni test.

References

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3. **Yu B, Wong MM, Potter CM, Simpson RM, Karamariti E, Zhang Z, Zeng L, Warren D, Hu Y, Wang W and Xu Q. Vascular Stem/Progenitor Cell Migration Induced by Smooth Muscle Cell-Derived Chemokine (C-C Motif) Ligand 2 and Chemokine (C-X-C motif) Ligand 1 Contributes to Neointima Formation. *Stem cells*. 2016;34:2368-80.**

Quantitative RT PCR primers sequences

GAPDH	CGACTTCAACAGCAACTCCCACTCTTCC	TGGGTGGTCCAGGGTTTCTTACTCCTT
ACTA2	TCCTGACGCTGAAGTATCCGAT	GGCCACACGAAGCTCGTTATAG
PDGFRβ	AGGAGTGATACCAGCTTTAGTCC	CCGAGCAGGTCAGAACAAAGG
CDH11	CTGGGTCTGGAACCAATTCTTT	GCCTGAGCCATCAGTGTGTA
COL1A	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGG
CD34	CCACAGACTTCCCCAACTG	CAGGCCTAACCTCAGACTGG
TET1	GCAGTGAACCCCGGAAAAC	AGAGCCATTGTAAACCCGTTG
TET2	AGAGAAGACAATCGAGAAGTCGG	CCTTCCGTACTCCCAAACTCAT
TET3	CACGGCTTCGAGGCAAGCCA	CCCCGGTTCCCATCCCCCAT
CDH5	AGGACAGCAACTTCACCCTCA	AACTGCCCATACTTGACC
TIE2	TCTGTGGAGTCAGCTTGCTCCTTT	TGAGGGATGTTTCGGCATCAGACA
FLK1	GGGATGGTCCTTGCATCAGAA	ACTGGTAGCCACTGGTCTGGTTG
FLI1	CTCCTACATGCCTTCCTACC	GTTTGGATAGATCCCAGCAG
EGFL7	CGCTGTGTCAATACTGTGGGA	GTTCTAGCACATCAACCCGAG
RASIP1	TCATGGAGCAGCTTACGGACT	GGGTGCGAGGAAAAACTTTCA
ROBO4	ACTCGGGGACCTATATGTGTATG	GGGATTCCTGGATAGACACCC
POSTN	CCTGCCCTTATATGCTCTGCT	AAACATGGTCAATAGGCATCACT
Flt1	CGGAAGCTCTGATGATGTGA	CTTCATGGAGGCCTTGG
IGFBP5	CCCTGCGACGAGAAAGCTC	GCTCTTTTCGTTGAGGCAAACC