Single-cell profiling reveals heterogeneity and functional patterning of GPCR expression in the vascular system

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G-protein-coupled receptor (GPCR) expression is extensively studied in bulk cDNA, but heterogeneity and functional patterning of GPCR expression in individual vascular cells is poorly understood. Here, we perform a microfluidic-based single-cell GPCR expression analysis in primary smooth muscle cells (SMC) and endothelial cells (EC). GPCR expression is highly heterogeneous in all cell types, which is confirmed in reporter mice, on the protein level and in human cells. Inflammatory activation in murine models of sepsis or atherosclerosis results in characteristic changes in the GPCR repertoire, and we identify functionally relevant subgroups of cells that are characterized by specific GPCR patterns. We further show that dedifferentiating SMC upregulate GPCRs such as Gpr39, Gprc5b, Gprc5c or Gpr124, and that selective targeting of Gprc5b modulates their differentiation state. Taken together, single-cell profiling identifies receptors expressed on pathologically relevant subpopulations and provides a basis for the development of new therapeutic strategies in vascular diseases.
-protein-coupled receptors (GPCRs) are the largest family of transmembrane receptors in eukaryotes; they transduce signals of numerous physicochemical stimuli including neurotransmitters, hormones, local mediators, metabolic or olfactory cues, and light. The human genome encodes ~800 GPCRs, the majority of them being olfactory receptors. Among the 367 non-olfactory GPCRs are still ~150 orphan receptors, that is, GPCRs for which ligand and function are still unknown.

GPCRs have regulatory functions in almost all organ systems, and dysregulation of GPCR signalling has been implicated in the pathogenesis of many diseases. The unique combination of diversity and specificity within the GPCR family, together with the fact that they are readily targetable by exogenous agonists and antagonists, has made GPCRs a most successful group of drug targets. In the vascular system, GPCRs modulate critical parameters such as vessel tone or endothelial permeability; they rely on the assumption that all cells of a given population are equal, a major limitation, since current interpretations of expression data suffer from the inability to resolve cell type-specific expression patterns and show examples of how this information can be used to modulate cell differentiation.

### Results

#### Array design and quality control
To narrow down the spectrum of GPCRs to be tested on the single-cell level, we determined GPCR expression in bulk RNA of vascular EC and SMC. Of 424 tested GPCRs, 122 GPCRs were expressed in at least one of these cell types (Supplementary Fig. 1A). For these 122 GPCRs, as well as for 32 additional GPCRs, a primer array for single-cell expression analysis was designed (Table 1). In addition, 13 genes

### Table 1 | Overview over the genes included in the array.

| Genes included | No. |
|----------------|-----|
| **GPCRs** | 154 (introm-spanning: 132) |
| - Adrenergic: Adra1a, Adra1b, Adra2d, Adrb1, Adrb2 | |
| - Chemokine: Ccbp2/Ackr2, Ccr10, Ccr1, Ccr2, Ccr3, Ccr4, Ccr5, Ccr6, Ccr7, Ccr8, Ccr9, Ccr11, Ccr2, Cx3cr1, Cxcr1, Cxcr2, Cxcr3, Cxcr4, Cxcr5, Cxcr6, Cxcr7, Cxcr1 | |
| - Lysophospholipid: Lpar1, Lpar2, Lpar3, Lpar4, Lpar5, Slpr1, Slpr2, Slpr3, Slpr4 | |
| - Miscellaneous: Adcyap1r1, Adora1, Adora2a, Agrp40, Agrp41, Agrp42, Bdkrb2, C3ar1, C5ar1, Ccrl2, Ccl3, Ccl4, Ccl5, Ccr5, Cx3cr1, Cxcr1, Cxcr2, Cxcr3, Cxcr4, Cxcr5, Cxcr6, Cxcr7, Cxcr1 | |
| - Orphan: Cd97, Celsr2, Darc, Etfal, Etn, Etnm, Gpr107, Gpr108, Gpr111, Gpr114, Gpr116, Gpr123, Gpr132, Gpr137, Gpr137b, Gpr137c, Gpr139, Gpr140, Gpr141, Gpr142, Gpr143, Gpr144, Gpr145, Gpr146, Gpr147, Gpr148, Gpr149, Gpr153, Gpr154, Gpr176, Gpr180, Gpr182, Gpr21, Gpr30, Gpr32, Gpr35, Gpr39, Gpr4, Gpr40, Gpr42, Gpr46, Gpr56, Gpr63, Gpr83, Gpr97, Gpr99, Gps3c, Gprcx1, Gprcx2, Lgr4, Lgr5, Lgr6, Lplnt1, Lplnt2, Lplnt3, Mgarph, Mhattan | |
| - Prostanoid: Ptger1, Ptger2, Ptger3, Ptger4, Ptger5, Ptgr1, Ptgr2, Tbx2a2r | |
| - Protease: F2r, F2r1, F2r2, F2r3 | |
| - Purinergic: P2y1l, P2y1o, P2y12l, P2y13, P2y14, P2y2, P2y6 | |
| - eGFp, Myh11 (smooth muscle), Cdhl (epithelial), Cdhn5 (endothelial), Ptprc (leukocyte), Itgama (myeloid), Ly6g (neutrophil), Cd19 (B lymphocyte), Cd44 (CD4 T lymphocyte), Lyve1 (lymphatic EC), Cspr4 (pericyte), Tmn2 (skeletal muscle) | |
| - Cell activation/differentiation: Cd25, Cd44, H2-Ab1, Pecam1, Acta2, Edn1, Dll4, Sele, Smmn, Icam1, Vcam1, Col1a2, Col3a1 | |
| - Cytokines/Growth factors: CsF2, CsF3r, Kd, Pdgfrb, Tgb1, Tnf, Ifng, Il10, Il17a, Il1b, Il2, Il6 | |
| - Transcription factors: Rorc, Cbx21, Mik67, Egr1, Fos, Hif1a, Hey2, Klf2 | |
| - Reference genes: Actb, Gapdh, Hprt | |
identifying individual cell types and 36 function-defining genes, including the three reference genes β-actin (Actb), glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and hypoxanthine phosphoribosyltransferase (Hpirt), were included in the array (Table 1). Whenever possible intron-spanning primer design was used, but for 22 single exon GPCRs this was not possible. These 22 non-intron-spanning primer pairs gave positive results in all tested cell types (Supplementary Fig. 1B,C), indicative of contamination with genomic DNA. GPCRs not allowing intron-spanning primer design were therefore excluded from the analysis (strikethrough in Table 1). Individual EC or SMC were pre-sorted by flow cytometry from enzymatically digested tissues of mice expressing enhanced green fluorescent protein (EGFP) in EC or SMC and subjected to single-cell isolation, cDNA synthesis and RT-PCR amplification using a microfluidic-based system. Various vascular beds were analysed, including aorta (ao), skeletal muscle vasculature (sk), lung (lu) or brain (br). All primer pairs included in the array produced amplification products with the predicted melting behaviour in at least one of three cell types tested (EC, SMC and leukocytes) (Supplementary Table 3). Despite stringent sorting criteria, single-cell analysis of individual EC or SMC showed 10–18% contamination with other cell types (Table 2). Only cells with clear identity ('Cdh5 only’, ‘Myh11 only’) and positive expression of reference genes Gapdh and Hpirt as quality control were included in further analyses (Fig. 1a). A systematic comparison of expression data obtained by bulk RNA analysis or single-cell RT-PCR in SMC from skeletal muscle vasculature (SMsk) showed that of 74 GPCRs undetectable in bulk RNA, 26 showed expression in individual SMsk cells (Fig. 1b, left). Of 11 GPCRs with uncertain result in bulk cDNA, all showed amplification in individual cells (Fig. 1b, middle). Of 37 GPCRs clearly expressed in bulk RNA, two were completely absent in individual cell analysis (Fig. 1b, right). These two GPCRs, Eltd1 and Gpr116, are known to be highly specific for EC23, indicating that these discrepancies are due to contamination of SMsk with EC.

GPCR heterogeneity in SMC. In individual aortal SMC (SMao) of healthy adult mice, GPCR expression was very heterogeneous (Fig. 2a). In total, 76 GPCRs were detected in SMao, but only 19 of them were expressed in more than 50% of cells, and only eight GPCRs (Lphn1, Lgr6, F2r, Adra1d, Cdh97, Gpr107, Gpr108 and Mrgprf) were expressed in more than 90% of cells (Fig. 2a). Reference genes Actb and Gapdh as well as SMC marker gene Myh11 were homogenously expressed (Fig. 2a, top). On average, individual cells expressed 20.3 ± 0.9 of the tested 132 GPCRs, though the individual values varied between 3 and 38 GPCRs per cell (Fig. 2b). mRNA sequencing of individual SMao showed even lower frequencies of GPCR expression (selected data in Supplementary Fig. 2, whole data set in Supplementary Data 1), which led us to verify single-cell expression data in GPCR reporter mice that are genetically engineered to express β-galactosidase (βgal) under control of different GPCR promoters. Flow cytometric analysis of βgal expression in freshly isolated SMao from Mrgprf-, Gabbr1- or Gprc5b-reporter mice closely matched the results of the single-cell RT-PCR expression analysis, while mRNA sequencing data underestimated GPCR expression (Fig. 2c,d). We also sequenced single-cell RT-PCR amplicons to exclude off-target amplification or amplification of highly homologous GPCRs and found that the amplified sequences were in all cases specific for the targeted receptor (Supplementary Fig. 3 and Supplementary Data 2). To investigate whether the same degree of heterogeneity was present on the protein level, we analysed expression of select GPCRs in individual SMao by flow cytometry. We found that also on the protein level GPCR expression was heterogeneous, and that the percentages roughly matched the values obtained by single-cell RT-PCR (Supplementary Fig. 4A,B). Ex vivo culture of primary SMao (passage 1) resulted not only in an upregulation of genes indicative of a dedifferentiated SMC phenotype, for example, intercellular adhesion molecule 1 (Icam1), vascular cell adhesion molecule 1 (Vcam1), marker of proliferation Ki-67 (Mki67) or interleukin-6 (Il6), but also in strongly increased expression frequency for the majority of GPCRs (Fig. 2e). Some GPCRs also showed decreased expression, such as Lgr6, Npy1r, Crhr2 or Bdkrb2 (Fig. 2e), but the average number of GPCRs per cell was still strongly increased (Fig. 2f). GPCR expression frequencies in cultured human aortal SMC (passage 1) matched in most cases the murine data (Fig. 2g middle), though some GPCRs showed notable difference between the species or between individual human donors (Fig. 2g right).

**Table 2 | Contaminating cells in single-cell expression analysis.**

| Cell type | Sorted for | Marker gene expression (%) |
|-----------|------------|-----------------------------|
|           |            | Cdh5 only | Myh11 only | Other markers | No marker |
| ECsk      | Cdh5-EGFP  | 81.5      | 0.0        | 17.8          | 0.7        |
| EClu      | Cdh5-EGFP  | 80.8      | 0.0        | 11.6          | 7.6        |
| ECbr      | Cdh5-EGFP  | 85.4      | 0.0        | 10.4          | 4.2        |
| SMao      | Myh11-EGFP | 0.0       | 81.5       | 15.2          | 3.2        |
| SMsk      | Myh11-EGFP | 0.8       | 84.0       | 12.0          | 3.2        |

Sorted EC and SMC were subjected to single-cell expression analysis and re-evaluated based on the expression of various identity-defining genes.

**GPCR repertoire in different types of SMC.** We next investigated whether the strong heterogeneity of GPCR expression was a special feature of conductance arteries such as the aorta, or whether it was also present in resistance arteries, for example, from SMsk. Also in SMsk GPCR expression was very heterogeneous (Fig. 3a), although the overall number of GPCRs per cell was higher than in SMao (Fig. 3a,b). The repertoire of GPCRs expressed in the two SMC types differed strongly: GPCRs Lphn2, Cmklr1, Lpar1, Gpr133, P2y6, Lgr6 and F2r were mainly present in SMao, while a large number of other GPCRs were predominantly expressed in SMsk (Fig. 3a). Among those receptors preferentially or selectively expressed in SMsk, the largest group were peptide hormone receptors (Fig. 3c), for example, receptors for endothelin (Ednra, Ednrb), angiotensin II (Agr1a), vasopressin (Avpr1a), neuropeptide Y (Npy1r), pituitary adenylate cyclase-activating polypeptide (PACAP, acting on Adyap1r1), vasoactive intestinal polypeptide (VIP, acting on Vipr1 and Vipr2), corticotropin releasing hormone (Crhr1), parathyroid hormone (Pth1r) or calcitonin gene-related peptide (CGRP, acting on Calcrl) (Fig. 3d). Also a number of chemokine and orphan receptors were preferentially expressed in SMsk (Fig. 3e). We also analysed GPCR expression in other SMC types, for example, from the mesenteric vasculature (SMmes) or urinary bladder (SMub). K-means cluster analysis revealed that SMC from mesenteric or skeletal muscle vasculature, two regions rich...
in resistance arteries/arterioles, were quite similar in their GPCR profile, but differed strongly from SMao or SMub (Fig. 3f,g). An analysis of differential gene expression for all SMC types is shown in Supplementary Fig. 5A–C; a comparison of GPCR frequencies in different types of EC is available in Supplementary Table 4.

**Endothelial GPCR pattern after acute inflammatory activation.** We next investigated how the GPCR repertoire changes in individual EC in response to acute inflammatory activation. To do so, we used a murine sepsis model induced by intraperitoneal (i.p.) injection of bacterial endotoxin lipopolysaccharide (LPS), which results in massive direct and indirect activation of ECs.24 ECbr showed 12 h after LPS injection a clear upregulation of markers of inflammatory activation such as *Icam1* or E-selectin (*Sele*), whereas expression of vascular endothelial growth factor receptor 2 (VEGFR2, encoded by *Kdr*) or endothelin-1 (*Edn1*) was reduced (Fig. 5a). Also the GPCR pattern changed significantly: numerous orphan receptors (*Gpr5a, Gpr97, Gpr111, Gpr153, Lphpn2, Gpr107, Gprc5b* and *Gpr56*) were upregulated, as well as select chemokine receptors (*Darc, Ccr2* and *Cxcr7*) and purinergic receptors (*P2ry2, P2ry6* and *Adora2a*). Other GPCRs, such as *Tbxa2r, Lphpn1, Gpr125, Gabbr1, Gpr124, Calcr or Cdc97*, were downregulated (Fig. 5a), resulting in total in a non-significant increase in the number of GPCRs per individual cell (Fig. 5b). K-means cluster analysis confirmed ECbrLPS as a distinct population characterized by a specific GPCR repertoire (Fig. 5c,d). Also in EClu characteristic changes in the GPCR expression pattern were observed upon LPS treatment, though with distinct differences to ECbr (select data in Fig. 5e,f; all data in Supplementary Fig. 6); the average GPCR number per cell was 161 (ECbrLPS) and 189 (ECblLPS). Smaller differences were observed in SMsk (Fig. 5g).

**GPCR heterogeneity in different types of EC.** To determine GPCR heterogeneity in primary murine EC from different locations, we isolated individual EC from lung, skeletal muscle or brain (EClu, ECsk, ECbr) (selected GPCRs in Fig. 4a, all data in Supplementary Fig. 6). Like SMC, EC showed a high heterogeneity of GPCR expression, with only five receptors (*Calcrl, Gpr116, S1pr1, Gpr30, Gpr124*, *Gpr4* or *Lpar4*) being homogenously expressed in all EC types (Fig. 4a, Supplementary Fig. 6). Individual EC from different vascular beds expressed on average 20.9, 21.0 and 16.3 of the tested 132 GPCRs (Fig. 4b). K-means cluster analysis identified three-cell clusters, which largely corresponded to the three different EC types (Fig. 4c) and are characterized by specific GPCR repertoires: receptors *Glp1r, Calcr, Lphpn3, Cebpb, Celsr2* and *Cdc97* were strongest expressed in cluster 1 cells (containing EClu) (Fig. 4d), whereas receptors *Darc, Ptgere4, P2ry6, Cyslrl1* or *Gprc5a* were strongest in cluster 2 cells (containing mainly ECsk) (Fig. 4e). Cluster 3 cells (mainly ECbr) showed higher expression of *Gpr30, Gpr124, Gpr4* or *Lpar4* (Fig. 4f).

**Figure 1 | Single-cell RT-PCR analysis in freshly isolated vascular cells.** EC or SMC obtained from skeletal muscle vasculature (sk), lung (lu), aorta (ao) of Cdh5-Cre; dTom/EGFP-reporterpos mice (Cdh5-EGFP) and tamoxifen-treated Myh11-CreERT2pos; dTom/EGFP-reporterpos mice (Myh11-EGFP), respectively, were subjected to single-cell RT-PCR. (a) Expression of identity-defining genes and quality control genes (*Gapdh, Hprt and Actb*) after exclusion of contaminating cells or marker negative cells (each dot one cell). (b) Comparison of expression data obtained by multiplex RNA expression analysis in pooled SMsk and single-cell RT-PCR (sc RT-PCR) in individual SMsk. GPCRs are arranged on the abscissa according to their expression strength as calculated in pooled RNA analysis, the ordinate shows the strength of gene expression in individual cells (each dot one cell). Green boxes indicate genes negative in sc RT-PCR but positive in pooled RNA analysis (cell/animal numbers: sc RT-PCR: *n* = 57 cells from seven mice; pooled RNA analysis: 500 ng from 10^6 cells per six mice. Values of genes that are not expressed were scattered around 0 to allow graphical estimation of cell counts). Expression data are calculated as follows: Gene expression = 2^((Ct - Ct) limit of detection (LoD) sample/LoD standard); LoD Ct is set to 24.
**Figure 2 | GPCR expression in individual aortal SMC (SMao).** (a) Heat map of GPCR expression in 60 SMao (each column one cell) from eight mice (sorted by frequency; horizontal bars on the right side visualize expression frequency (in %); frequencies <10% not shown (for full data set, Supplementary Fig. 6); expression of Myh11, Actb and Gapdh as quality control). (b) Number of GPCRs expressed in individual SMao. (c) Example of flow cytometric analysis of βgal-positive SMao in control mice and Gabbr1-lacZ reporter mice. (d) Comparison of GPCR expression frequency in individual cells as judged by single-cell RT-PCR (sc RT-PCR), single-cell mRNA sequencing (sc mRNAseq), and flow cytometric analysis of reporter-positive SMao from MgrpF/Gabr1/-/Gpr5b-reporter mice (FACS reporter, n = 3 per group). (e) Percentage of cells expressing selected genes in freshly isolated SMao (n = 60) and SMao cultured for one passage (7–10 days) (SMao_cul; n = 29). (f) Average number of GPCRs expressed in freshly isolated SMao and SMao_cul. (g) Percentage of cells expressing a given gene in cultured murine (SMao_cul) and human (huSMao_cul) aortal SMC (#1: 2-year-old healthy male child, n = 42 cells; #2: 51-year-old male adult, n = 31 cells); Expression data are calculated as follows: Gene expression = 2^(-ΔΔCt, Limit of detection (LoD) Ct—sample Ct); LoD Ct was set to 24. Data in b,d,f are shown as mean ± s.e.m.; comparisons between groups were performed using two-tailed t-test; ***P < 0.001.
as separate clusters (Fig. 5h). Detailed analysis of transcriptional changes showed that both EC types showed upregulation of Icam1, Vcam1 and Sele, whereas Kdr, platelet-derived growth factor b (Pdgfb), and Edn1 were downregulated (Supplementary Fig. 7A). Both EC types showed upregulation of GPCRs Ccr12, Cxcr7, Gpr111 and others (Supplementary Fig. 7B), whereas Cacrl, Cd97, Gabbr1, Lphn1 and so on were downregulated (Supplementary Fig. 7C). However, some GPCRs were differentially regulated: Ptger2, P2ry10, P2rl1, Cebpb, Glp1r, Celsr2 or Cxcr4 were up- or downregulated selectively in EClu (Fig. 5i), while Gpr107, Gpr97, Adora2a, Gpr153, Darc, Fpr2, C5ar1 and Ccr1 were only upregulated in ECbr (Fig. 5j).

Interestingly, upregulation of Fpr2, C5ar1 and Ccr1 was restricted to a small subpopulation of ECbrLPS that showed at the same time reduced expression of GPCRs such as Darc, Gpr97, Gabbr1 and others (Fig. 5k). Though expression of Fpr2, C5ar1 and Ccr1 is normally restricted to myeloid cells (Supplementary Fig. 6), this subpopulation was clearly positive for Cdh5, Pecam1 and EC-specific GPCRs such as Gpr116 or Eltd1, but negative for leukocyte markers Ptprc, Itgam and Ly6g (Fig. 5k), indicating they are indeed an EC population that assumed a myeloid-like GPCR expression pattern.

To also assess the endothelial GPCR repertoire under conditions of chronic vascular inflammation, we investigated aortal EC from ApoE-deficient mice kept for 16 weeks on a Western-style high-fat diet25. Surprisingly, all Cdh5-positive EC obtained from aortae were also positive for SM marker Myh11, although Myh11 levels were very low compared to normal SMC
conditions (Fig. 6f). Interestingly, a number of GPCRs were upregulated both in acute and chronic inflammation, whereas expression of adhesion molecules and inflammatory activation such as Icam1, Cxcr7, Gpr56, Gprc5a and others (Fig. 6f).

**Figure 4 | Single-cell GPCR expression in different EC types.** (a) Heat map of GPCR expression in EClu, ECsk, ECbr (48, 40, 52 cells per EC type, from six to eight mice per group); horizontal bars on the right side visualize expression frequency in % (for full data sets, Supplementary Fig. 6).

(b) Average number of GPCRs expressed in individual EC types (mean ± s.e.m.). (c) t-SNE plot of k-means clustering data for different EC types: cluster assignment is indicated by coloured numbers, cell type is indicated by symbol (each dot one cell; distance between dots indicates degree of similarity). (d-f) Genes differentially expressed in cluster 1 (EClu, d), cluster 2 (mainly ECsk, e), and cluster 3 (mainly ECbr, f) (only genes significantly regulated (P < 0.05) and with fold change > 0.7 or > 1.5 are displayed). Data in b are mean ± s.e.m.

**GPCR repertoire in SMC from atherosclerotic mice.** We next studied GPCR patterns in SMC from atherosclerotic aortae. After 16 weeks of high-fat diet, SMao showed clear signs of inflammatory activation and dedifferentiation26,27: Icam1, Vcam1 and Il6 were upregulated, whereas contractile proteins such as Acta2 and Myh11 were reduced in expression strength, though not in frequency (Fig. 7a, upper part). In addition, numerous SMao showed increased expression frequency (Fig. 7a, lower part), resulting in a significantly increased number of GPCRs per individual cell (Fig. 7b). K-means cluster analysis assigned the majority of SMao from atherosclerotic mice (squares in Fig. 7c) to a cluster characterized by increased expression of GPCRs such as Olfr78, Ednrb, Adcyap1r1, Ptgir2, Avpr1a1a, S1pr3, Ptgir, Vipr2, Ccr12, Cxcr7, Gprc5b or Gprc5e (Fig. 7d). ApoE-deficient mice without high-fat diet showed an intermediate phenotype, but no clear upregulation of inflammatory genes such as Icam1 or Vcam1; comparable changes were observed in aged but otherwise healthy C57BL/6 mice (Fig. 7e). To understand the mechanisms regulating GPCR expression in healthy and dedifferentiating SMao, we analysed transcription factor (TF) binding sites in promoters of GPCRs that were either up- or downregulated in dedifferentiating SMC. GPCRs that were upregulated in atherosclerotic SMao were more likely to contain binding sites...
for TFs such as heat shock factors proteins 1,2,4 (HSF1,2,4), retinoic acid receptor α/β (RARA, RARB), NF-κB2, AP2 (TFAP2A,B,C), KLF5 and others (Fig. 7f, left side). GPCRs that were downregulated in atherosclerotic SMao were more likely to contain bindings sites for, among others, estrogen-related nuclear receptors ESRRA and ESRRB, sterol regulatory element-binding transcription factor 1 (SREBF1), or T box TFs TBX1 and TBX19 (Fig. 7f, right side; complete list in Supplementary Table 5).
Dedifferentiating SMC of the healthy aorta. We noticed that some Smao from healthy mice were assigned to the cluster of dedifferentiating SMC in Fig. 7c (marked by arrows), indicating that a spontaneously dedifferentiating population exists in the healthy aorta. Indeed, k-means analysis of Smao from healthy mice identified a small subpopulation of cells as being clearly different from the rest (cluster 3 in Fig. 8a). These cells were characterized by a reduced expression of contractile proteins Myh11 or Acta2, but increased expression of Icam1, Vcam1, Col1a2 and Col3a1 (Fig. 8b). This dedifferentiated phenotype was associated with reduced expression of GPCRs such as Lgr6 and Adra1d, and increased expression of Ptgir, Vipr2, Gpr39, Lpar1, Gprc5b, Gpr124, Cxcr7, Gpr137b, Ednra and others (Fig. 8b). Spearman’s rank correlation analysis confirmed the positive correlation between Myh11, Acta2, Lgr6, Adra1d on the one hand and Icam1, Vcam1, CD44, Ptgir, Gpr39, Vipr2, Cxcr7, Gpr5b and so on on the other hand (Fig. 8c). A direct comparison of changes in dedifferentiating SMC from healthy mice and atherosclerotic mice showed that both cell types behaved similarly with respect to upregulation of adhesion molecules and GPCRs such as Ptgir, Vipr2, Gprc5b, Gpr5c, Cxcr7 and so on, as well as down-regulation of Acta2, Myh11, Adra1d and Lgr6 (Fig. 8d, left side). However, a number of GPCRs were upregulated only in the context of atherosclerotic dedifferentiating, and not in spontaneous dedifferentiation, such as Olfr78, Ednrb, Adcyap1r, Ptgir and others (Fig. 8d, right side).

Since vascular SMC dedifferentiation is believed to occur mainly in regions of disturbed flow, for example, the inner curvature of the aortic arch2,8, we used Gprc5b-ßgal reporter mice to investigate the localization of spontaneously dedifferentiating SMC in vivo. Immunohistochemical analysis of the expression of ßgal and SMC marker ç-smooth muscle actin (çSMA) in transverse section of the aortic arch (Fig. 8e) showed only sparse ßgal expression in SMC of the outer curvature, but an enrichment of ßgal/çSMA-positive cells in the inner curvature (Fig. 8f,g). These data confirm the notion that GPCRs such as Gprc5b are selectively expressed in SMC in atheropane regions. We finally investigated whether selective targeting of GPCRs that are specifically expressed in dedifferentiating Smao may be used to modulate their functional state. Knockdown of Gprc5b resulted in freshly isolated Smao from ApoE-deficient mice in a significant increase in expression of Icam1 and Ile6, indicating that this receptor plays a modulatory role in inflammatory gene expression in dedifferentiating SMC (Fig. 8h).

**Discussion**

We report here the analysis of GPCR expression in primary vascular cells on the single-cell level. Compared to conventional expression analysis in pooled cell RNA/cDNA, single-cell expression analysis has three crucial advantages: First, it allows to rigorously exclude contaminating cells and thereby precludes misinterpretation of data. Second, single-cell expression analysis is able to detect transcripts in rare cell populations that might be below threshold in bulk RNA/cDNA analyses: 37 GPCRs that were judged negative or uncertain in Ssk by NanoString analysis were detected in individual Ssk, though in some cases only in 1.5% of cells. However, due to the limited amount of RNA obtained from an individual cell also single-cell expression may overlook very low abundance transcripts, in particular if the RNA quality is compromised. Since GPCRs are in many cases expressed at low levels29, we directly compared the two major readout systems for single-cell expression analysis, RT-PCR and mRNA sequencing, with respect to their sensitivity for GPCR detection in Smao. RT-PCR detected most GPCRs with higher frequency than mRNA sequencing, and sequencing of the single-cell amplificates as well as analyses in GPCR reporter mice and on the protein level largely confirmed the RT-PCR data. A possible explanation for this difference in sensitivity lies in the fact that single-cell RT-PCR uses target-specific pre-amplification of mRNAs, while pre-amplification for mRNA sequencing is unbiased.

The third and probably most relevant advantage of single-cell expression analysis is that it allows to estimate the degree of GPCR heterogeneity within a cell population and, consecutively, to identify correlations between GPCR profile and functional state of a given cell. We found that all types of SMC and EC showed a surprisingly high heterogeneity of GPCR expression, and reporter analysis, as well as studies on the protein level confirmed these findings. Studies in other fields, mainly developmental biology, suggest that this is not a specialty of GPCRs, but is also observed in other gene families17,18. These findings have major implications for pharmacotherapy, since current interpretations of GPCR expression data rely on the assumption that all cells of a given population are equal, or at least comparable15,16,30,31. Our data not only clearly disprove this assumption for the majority of GPCRs, they also open up the possibility to selectively target pathologically altered cells based on their specific GPCR repertoire.

We show, for example, that dedifferentiated Smao differ from normal Smao not only in the expression of typical markers indicating inflammatory activation and dedifferentiation, but also in their GPCR repertoire. Among those receptors that are preferentially expressed in dedifferentiating Smao are a number of Gs-coupled receptors with known anti-inflammatory and anti-proliferative properties, such as the prostacyclin receptor Ptgir or the VIP receptor Vipr2 (refs 32,33). It is tempting to speculate that also other Gs-coupled receptors upregulated in dedifferentiating Smao will exert anti-proliferative effects, for example, the PACAP receptor Adcyap1r1, or the adenosine

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**Figure 5** | Endothelial GPCR pattern after acute inflammatory activation by LPS *in vivo*. (a-d) Analyses in brain EC (ECbr): (a) Heat map of GPCR expression in ECbr from healthy mice and LPS-treated mice (ECbrLPS) (52 and 22 cells from seven and four mice, respectively). Horizontal bars on the right side visualize expression frequency (in %) for full data set, Supplementary Fig. 6); function-defining genes are shown in blue. (b) Average number of GPCRs expressed in individual ECbr from healthy or LPS-treated mice. (c) Heat map indicating dissimilarities between individual ECbr. K-means clustering identified two cell clusters that are colour-coded along the axes and correspond to ECbr from healthy and LPS-treated mice, respectively. (d) Average number of GPCRs detected in individual ECbrLPS compared to all cells. (e–g) Analyses in lung EC (EClu) (48 and 25 cells from eight and four mice, respectively): (e) Heat map indicating dissimilarities between individual EClu. K-means clustering identified two cell clusters that are colour-coded along the axes and correspond to EClu from healthy and LPS-treated mice, respectively. (f) Fold difference in gene expression in ECluLPS compared to all cells. (g) Average number of GPCRs expressed in individual EClu from healthy or LPS-treated mice. (h–j) Comparison of LPS effects in EClu and ECbr: (h) T-SNE plot of k-means clustering data for different EC types with and without LPS treatment: cluster assignment is indicated by coloured numbers, cell type is indicated by symbol (each dot one cell; distance between dots indicates degree of similarity). (i,j) Comparative analysis of expression strength of selected GPCRs in different EC types. (k) Rearranged and extended heat map of ECbrLPS shown in a: Fpr1/Fpr2/Cortis/Csr1l-expressing cells are indicated by red box. All expression data are calculated as 2^[-1/2(Log2 expression) / standard deviation] (t-test; *P < 0.05; **P < 0.01; ***P < 0.001).
**Figure 6 | Endothelial GPCR pattern in atherosclerosis.** (a) Average expression strength of SM marker Myh11 in SMC from aorta (SMao) or skeletal muscle vasculature (SMsk), or in Cd31-positive aortal cells. (b) Percentage of cells expressing selected GPCRs in freshly isolated EC from skeletal muscle (ECsk) or brain (ECbr), as well as in Cd31<sup>pos</sup>, Myh11<sup>low</sup> aortal cells or aortal SMC (SMao). (c) Heat map of GPCR expression in aortal EC from healthy mice (ECao) and ApoE-deficient mice kept for 16 weeks on high-fat diet (ECaoApo16w) (12 and 16 cells from four to six mice, respectively). Horizontal bars on the right side visualize expression frequency (in %) (for full data set, Supplementary Fig. 6). (d) Comparative analysis of gene expression strength in ECao and ECaoApo16w. (e) Average number of GPCRs expressed in individual ECao from healthy and atherosclerotic mice. (f) Changes in endothelial gene expression in response to acute inflammatory activation by LPS or chronic inflammatory activation in atherosclerotic mice (n.e., not expressed). All expression data are calculated as 2<sup>(limit of detection (LoD) Ct—sample Ct)</sup>; LoD Ct was set to 24. Function-defining genes are shown in blue. Data in a,d,e, are means ± s.e.m; comparisons in d,e were performed using two-sample t-test. *P<0.05; **P<0.01.
receptor Adora2a. In line with this notion, PACAP was shown to inhibit SMC proliferation and enhanced SMC proliferation was observed in Adora2a-deficient mice. Furthermore, it will be particularly interesting to investigate whether those orphan receptors that are upregulated in dedifferentiating SMC, for example, Gpr39, Gpr124, Gpr153 or Gprc5b have the potential to positively or negatively regulate SMC differentiation. In support of this idea, we found that knockdown of Gprc5b enhanced proinflammatory gene expression in freshly isolated SMao, suggesting that this receptor negatively modulates inflammatory gene expression in SMC.

How the GPCR repertoire of an individual vascular cell is shaped and how stable it is over time, is unclear. While numerous studies analysed the posttranslational regulation of GPCRs by phosphorylation, internalization or dimerization, their transcriptional control is little understood. To address the mechanisms regulating GPCR expression in healthy and dedifferentiating SMao, we analysed TF binding sites in promoters of GPCRs upregulated or downregulated in dedifferentiating SMao (cluster 1 from c) compared to a global promoter background set. Colour scale represents a rank based z score; for full list see Supplementary Table 5. Data in b are mean ± s.e.m.; comparisons were performed using two-sample t-test. ***P < 0.001.
Figure 8 | Functional subgroups within SMao. (a) Heat map indicating similarities/dissimilarities between 60 individual SMao. Cell clusters identified by k-means clustering are colour-coded along the axes. (b) Heat map of GPCR expression in SMao (cells sorted from left to right by clusters shown in a). (c) Graphical representation of Spearman’s rank correlation coefficients calculated for selected genes expressed in SMao (width of connecting line indicates strength of correlation). (d) Changes in gene expression in dedifferentiating SMC from healthy aortae or atherosclerotic aortae. (e–g) Immunohistochemical analysis of βgal and αSMA expression in transverse sections of the aortic arch of Gprc5b-βgal reporter mice (schematic diagram in e, exemplary photomicrographs in f, quantification in g). (h) Gene expression in SMao cultured for 7 days after knockdown of Gprc5b (normalized to Actb expression). Data in g,h are shown as mean ± s.e.m.; comparisons were performed using two-sample t-test (g) or one-sample t-test (h) (n = 4–6). *P < 0.05.
atherosclerotic vessels, and NF-κB activation enhances expression of inflammatory genes such as Vcam1 and reduces expression of SM-specific genes. These data suggest that TFs implicated in the regulation of SMC dedifferentiation also control changes in the GPCR repertoire, which in turn might modulate the dedifferentiation process.

Our data also show that both SMC and EC express distinct GPCR repertoires depending on the vascular bed they originate from. SMC from vascular beds rich in resistance arteries, here SMsk and SMmes, express significantly more GPCRs than aortic SMC, in particular more peptide hormone receptors and orphan receptors. Most of the peptide hormones in question have been shown to affect vascular tone: vasorelaxation is, for example, mediated by the gastrointestinal hormones VIP and PACAP which are known agonists/antagonists so far no data are available with targets for modulation of blood pressure, but due to the lack of known agonists/antagonists so far no data are available with respect to their function in regulation of vascular tone or other SMC functions.

Also EC show, depending on their anatomical location, remarkable differences in the GPCR repertoire, and also their responses to acute inflammatory activation differ. Brain EC, for example, were characterized by upregulation of chemokine receptor Darc and orphan receptor Gpr153, a response that was absent in lung EC. In line with this finding it was recently shown that DARC is upregulated in brain EC during neuro-inflammation, where it shuttles inflammatory chemokines across the blood-brain barrier. The role of GPR153 in activated brain EC, in contrast, is completely unclear. Interestingly, we also detected a small subgroup of brain EC that were characterized by a myeloid-like GPCR expression pattern, but were devoid of any myeloid lineage markers. The function of this subpopulation is currently unclear, but the fact that expression of these GPCRs was negatively correlated with expression of Icam1 or chemokine receptors involved in leukocyte trafficking, such as Darc or Cxcr7(ref. 50), suggests that this population is less supportive of leukocyte extravasation than other LPS-activated ECs. It is also interesting to note that chronic inflammatory activation during atherosclerosis results in other changes than acute activation by LPS, for example, with respect to atypical chemokine receptor Cxcr7, or orphan GPCRs Gpr111, Gpr107, Gprc5a and Gprc5b. It is also noteworthy that ECa0 from atherosclerotic aortae showed reduced expression of endothelial genes such as Cd31, Pecam1, Gpr116 or Gpr56, suggesting that EC, very much like SMC, undergo dedifferentiation in atherosclerotic aortae in vivo.

Taken together, our results show that expression profiling on the single-cell level allows the identification of receptors that are predominantly or selectively expressed on pathologically relevant vascular subpopulations. Understanding the GPCR profile of these cells will not only significantly enhance our knowledge about the pathomechanisms of disease, it will also allow a more selective pharmacological targeting of these cells.
To investigate the potential role of transcription factors (TFs) in cell type-specific GPCR sets, we utilized the pscan tool. We investigated the promoter region of GPCRs within a given set at a size of ~950 to +50 bp around the respective transcription start site and tested for significantly (P<0.05) overrepresented/underrepresented TF binding sites listed in the current Jaspar 2016 release (http://jaspar.genereg.net) compared to a global promoter set of the mouse genome. Using P values and Fisher’s test, genes were investigated in a multi column heat map generated by the heatmap2 R function (package gplots, version 3.01).

Single-cell mRNA sequencing and expression analysis. Single-cell transcriptome analysis was performed on a C1 AutoPrep station (Fluidigm) using 5–10 µm mRNA arrays and standard protocols. In total, 15,000 events sorted on a JSAN swift sorter were loaded in 10 µl resuspension buffer (PBS + 0.5% bovine serum albumin + 2 nM EDTA, sterile filtered) and further diluted (6x4) in suspension buffer. A volume of 3 µl was loaded to the C1 Array while remaining cells (~10,000) were used for total RNA isolation using µRNeasy micro kit (Qiagen) combined with on-column DNase digest (Qiagen). RNA was quantified by Qubit HS RNA Assay (Thermo Fisher) and used for tube control. Sequencing was performed on NextSeq500 Sequencer (Illumina) using v2 chemistry and High Output Flow Cell with 75 bp single end protocol. Raw data files were trimmed by subtracting the mean of the positive control spike count, then a background correction was done followed by incubation of single-cell suspensions with PE- or FITC-labelled antibodies directed against 45 MA (ThermoFischer, S32357) and goat anti-chicken IgG (H + L)–Alexa-647 (ThermoFischer, A11041) (both 1:200). 4`-diamidin-2-phenylindol (DAPI) was used to label cell nuclei.

Flow cytometric analysis. β-galactosidase expression in murine aortic SMC was analysed using the FlowJo software and Flow cytometry kit (ThermoFischer) with propidium iodide to identify viable cells and anti-siRNA antibody (eosinio, #59376800) to identify SMC. For antibody-mediated detection of GPCR expression, sort cells were digested, fixed in 0.1% formaldehyde and permeabilized using FACS buffer (0.5% v/v in PBS), followed by incubation of resulting single-cell suspensions with PE- or FITC-labelled antibodies directed against 45 MA (ebsciences) and APC-labelled antibodies directed against receptors Cmklr1 (Milteny), Cfr2 (R&D), Cxcr7 (R&D), Celsr2 (R&D) in (all cases 1 µg antibody per 10⁶ cells per 200 µl). For each GPCR-specific antibody the corresponding isotype control was used. Analyses were performed on a FACS Canto II (Becton Dickinson) and data were analysed by Flowjo software (Tree Star).

General statistical analyses. Data are presented as means ± s.e.m. if not otherwise indicated. Comparisons between two groups were performed using two-sample t-test; normalized data (control group set to 1) were analysed by one-sample t-test. P values are indicated as follows: *P<0.05; **P<0.01; ***P<0.001.

Data availability. Single-cell RNA-Seq data have been deposited in GEO database (https://www.ncbi.nlm.nih.gov/geo, under the accession code GSE97955). Full data sets generated during the current study are available from the corresponding author on reasonable request.

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Author Correction: Single-cell profiling reveals heterogeneity and functional patterning of GPCR expression in the vascular system

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Correction to: Nature Communications https://doi.org/10.1038/ncomms15700; published online 16 June 2017

The original version of this Article omitted the following from the Acknowledgements:

‘This project was supported by CRC128/Project A03 of the Deutsche Forschungsgemeinschaft (DFG).’

This has not been corrected in either the PDF or HTML versions.

Published online: 28 March 2019

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