A role of stochastic phenotype switching in generating mosaic endothelial cell heterogeneity

Lei Yuan1,2,*, Gary C. Chan1,2,* , David Beeler1,2, Lauren Janes1,2, Katherine C. Spokes1,2, Harita Dharaneeeswaran1,2, Anahita Mojiri3, William J. Adams4, Tracey Sciuto1,4, Guillermo Garcia-Cardeña5, Grietje Molema6, Peter M. Kang2,7, Nadia Jahroudi3, Philip A. Marsden8,9, Ann Dvorak1,4, Erzsébet Ravasz Regan1,2,* & William C. Aird1,2

Previous studies have shown that biological noise may drive dynamic phenotypic mosaicism in isogenic unicellular organisms. However, there is no evidence for a similar mechanism operating in metazoa. Here we show that the endothelial-restricted gene, von Willebrand factor (VWF), is expressed in a mosaic pattern in the capillaries of many vascular beds and in the aorta. In capillaries, the mosaicism is dynamically regulated, with VWF switching between ON and OFF states during the lifetime of the animal. Clonal analysis of cultured endothelial cells reveals that dynamic mosaic heterogeneity is controlled by a low-barrier, noise-sensitive bistable switch that involves random transitions in the DNA methylation status of the VWF promoter. Finally, the hearts of VWF-null mice demonstrate an abnormal endothelial phenotype as well as cardiac dysfunction. Together, these findings suggest a novel stochastic phenotype switching strategy for adaptive homoeostasis in the adult vasculature.

DOI: 10.1038/ncomms10160

1 Center for Vascular Biology Research, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215, USA. 2 Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215, USA. 3 Department of Medicine, University of Alberta, Edmonton, Alberta T6G 2R3, Canada. 4 Department of Pathology, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215, USA. 5 Center for Excellence in Vascular Biology, Department of Pathology, Brigham and Women’s Hospital, Boston, Massachusetts 02215, USA. 6 Department of Pathology and Medical Biology, Medical Biology Section, University Medical Center Groningen, University of Groningen, 9700 AB Groningen, The Netherlands. 7 Cardiovascular Institute, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215, USA. 8 Department of Medicine, University of Toronto, Toronto, Ontario M5G 2C4, Canada. 9 St. Michael’s Hospital, Toronto, Ontario M5B 1W8, Canada. * These authors contributed equally to this work. † Present address: Biochemistry and Molecular Biology, The College of Wooster, Wooster, Ohio 44691, USA. Correspondence and requests for materials should be addressed to E.R.R. (email: eregan@wooster.edu) or to W.C.A. (email: waird@bidmc.harvard.edu).
Endothelial cells from veins and arteries and from capillaries of different organs demonstrate heterogeneity at structural, functional and molecular levels. Intriguingly, phenotypic heterogeneity also exists between neighbouring endothelial cells exposed to the same extracellular environment. For example, we, and others, have shown that von Willebrand factor (VWF), a circulating glycoprotein that mediates platelet adhesion to the subendothelial surface of injured blood vessels, is expressed in a mosaic pattern in the aorta and in selected capillary beds. It is widely held that phenotypic heterogeneity is driven by deterministic mechanisms, including hardwired cellular responses to the extracellular environment and/or epigenetic memory. However, recent evidence points to a role for biological noise in generating mosaic expression among isogenic cells. Noise is all-pervasive in cells, arising from omnipresent random fluctuations in the normal kinetic of molecular processes such as transcription and translation. Noise typically results in a normal ‘spread’ in gene and protein expression around the mean, as illustrated on the quasi-energy landscape in Fig. 1a (refs 4,11,21,22). In multistable regulatory systems, however, noise can drive transitions between distinct, locally stable states (Fig. 1b), converting a graded response to a binary response. Previous studies in prokaryotes and single-cell eukaryotes have implicated an adaptive role for biological noise in bet hedging and in task sharing/allocation. In higher eukaryotes, noise has been shown to trigger random cell fate decisions during development. However, to date, there is no evidence that stochastic phenotype switching occurs in differentiated cells within mammals, or that it plays a role in adult homeostasis.

In this study, we hypothesized that VWF mosaicism is driven by dynamic noise-induced transitions between ON and OFF VWF states. To test this hypothesis, we developed genetically modified mouse models that allow for a comparison of VWF expression in a single snapshot in time with its cumulative expression over time (Fig. 1c,d). We show that VWF flickers ON and OFF in the endothelium of some, but not all vascular beds. Using in vitro cell culture, we demonstrate that dynamic VWF mosaicism is generated by the ON/OFF toggle of a low-barrier, noise-sensitive bistable switch, and that it involves random transitions in VWF promoter DNA methylation. Finally, we present data that supports a role for mosaicism in endothelial health. Collectively, these findings suggest that biological noise is exploited by selected vascular beds to generate adaptive neighborhood-level phenotypic diversity, providing new insights into mechanisms of endothelial cell heterogeneity.

Results

VWF is expressed in a mosaic pattern in vivo. Previous studies have shown that VWF expression in the endothelium is higher in veins compared with arteries and in large vessels compared with capillaries. In immunofluorescent co-localization studies of VWF and the pan-endothelial marker CD31, we found that VWF expression is also heterogeneous at the level of single vessels. For example, VWF protein was detected in a minority of endothelial cells in the capillaries of adult heart, skeletal muscle, lung and brain (Fig. 2a shows heart). Consistent with previous reports, the aorta also consisted of micro-domains of VWF-positive and -negative endothelial cells (Fig. 2b). In contrast to these mosaic patterns, VWF expression was ubiquitous in venous endothelium (Fig. 2c shows heart), and virtually undetectable in kidney glomeruli and liver sinusoids (Fig. 2d shows liver). We previously targeted the lacZ reporter gene to the endogenous mouse Vwf locus (Supplementary Fig. 1a), and showed that expression of the reporter gene in heterozygous mice (VWF(+/−)lacZ) mirrored that of the endogenous gene. Indeed, LacZ expression followed a similarly mosaic pattern in the capillaries of heart, skeletal muscle, lung and brain, as well as in the aorta (Fig. 2a,b; Supplementary Fig. 2). Together, these findings indicate that at any point in time, VWF expression varies not only between organs and blood vessel types, but also between neighbouring endothelial cells.

Mosaic VWF expression is dynamic in selected vascular beds. The finding of mosaic expression raised the question of how the pattern plays out over time. If we were to ‘roll the film’, would the mosaic remain static, indicative of developmentally locked-in hotspots of VWF activity? Alternatively, does expression toggle ON and OFF in time asynchronously between individual endothelial cells? An ideal approach for confirming such dynamics would be to directly observe VWF protein (or a fluorescent reporter) in real time in the living organism. However, such a strategy is not feasible given current limitations in the resolution of intravital microscopy, combined with the need for potentially long windows of observation. Thus, we used an indirect approach to capture the temporal dynamics of VWF expression. Specifically, we generated mice in which Cre recombinase was targeted to the endogenous Vwf locus. These animals (VWF(Cre+/-)) were crossed with the ROSA26R reporter mouse line, which contains a LoxP-flanked STOP cassette upstream of the lacZ gene in the constitutively active Rosa26 locus. In double transgenic offspring (vWF–Cre–ROSA26R; Supplementary Fig. 1b), cells that express vWF–Cre (even transiently) excise the stop codon in the reporter allele, leading to permanent and heritable expression of LacZ. Thus, reporter gene activity reflects cumulative expression over the lifetime of the animal, and a comparison of LacZ expression between VWF(+/−) lacZ and VWF–Cre–ROSA26R mice provides critical information about the temporal dynamics of VWF expression (Fig. 1c,d).

Remarkably, adult vWF–Cre–ROSA26R mice demonstrated uniform LacZ staining in the capillaries of heart, skeletal muscle, lung and brain (Fig. 2a,c–g; Supplementary Fig. 2), suggesting that virtually every capillary endothelial cell had expressed LacZ (hence VWF) at some point during the lifetime of the animal. Since only a minority of these endothelial cells are positive at a single point in time, the data suggest that VWF flickers ON and OFF over time. Interestingly, LacZ expression in the aorta of adult vWF–Cre–ROSA26R mice remained patchy (Fig. 2j; Supplementary Fig. 3), suggesting that the mosaic pattern in this blood vessel is static. Finally, LacZ staining was undetectable in kidney glomeruli and liver sinusoidal endothelial cells (Fig. 2l shows liver), indicating that VWF is permanently OFF at these two sites. Together, the data support the notion that VWF toggles between ON/OFF states in some, but not all vascular beds. This behaviour is consistent with the existence of a bistable switch whose state-switching dynamics vary across the vascular tree, as illustrated by the quasi-energy landscapes in Fig. 2m–p.

VWF mosaicism is dynamically regulated in adult capillaries. The comparative analysis of VWF(+/−)lacZ and vWF–Cre–ROSA26R mice does not prove that VWF mosaicism is dynamic in the postnatal period. It is formally possible that the uniform LacZ staining in capillaries of adult vWF–Cre–ROSA26R mice reflects a developmental window in which a common ancestor of all endothelial cells expresses VWF. To rule this out, we monitored LacZ expression in vWF–Cre–ROSA26R mice from birth to adulthood. Analysis of tissues at postnatal days 1, 4 and 7 and in adult mice revealed a time-dependent LacZ accumulation in the capillary beds of heart, skeletal muscle, lung and brain (Fig. 3a shows heart and diaphragm). In contrast, a similar binary mouse model in which the pan-endothelial Tie2 promoter
drives Cre expression demonstrated uniform LacZ staining of blood vessels in both newborn and adult animals (Supplementary Fig. 4a–f).

To explicitly demonstrate that transitions between VWF-positive and -negative cells take place in adult tissues, we generated a mouse model in which we could control the timing of Cre recombinase expression from the Vwf locus. Specifically, we targeted tamoxifen-inducible Cre recombinase to the endogenous Vwf locus (vWFCreERT2/+) and crossed these mice with the ROSA26R reporter line (Supplementary Fig. 1c). Double-transgenic mice (vWF-CreERT2-ROSA26R) were treated with tamoxifen for increasing lengths of time (up to 4 weeks), and their organs were harvested for LacZ staining. In the absence of tamoxifen, there was no detectable reporter gene activity. In contrast, mice treated with tamoxifen demonstrated time-dependent LacZ accumulation in veins and capillaries of the heart, skeletal muscle, lung and brain (Fig. 3b shows diaphragm). Again, the aorta demonstrated a fixed mosaic, while there was no detectable expression in the kidney glomeruli or hepatic sinusoids (Supplementary Fig. 4g–i). These data indicate that uniform LacZ staining in adult muscle capillaries reflects the accumulation of transiently expressing (VWF ON/OFF) cells in the postnatal period, and that vascular bed-specific dynamic mosaicism is a property of healthy adult endothelium.

Dynamic VWF mosaicism is driven by biological noise in vivo. A simple explanation for spontaneous toggling between VWF ON
and OFF is biological noise. According to this hypothesis, the barrier of the binary switch is sufficiently low to allow for stochastic transitions in the capillaries of heart, skeletal muscle, lung and brain (Fig. 2m), yet high enough to preclude such transitions in the aorta (which displays a static ON/OFF mosaic; Fig. 2n)\(^4\). If dynamic VWF expression is indeed driven by biological noise with constant average strength, then the number of LacZ-negative cells in vWF–Cre–ROSA26R heart capillaries should decrease exponentially with time: \(P_{\text{OFF}}(t) \sim \exp(-(P_{\text{ON}} \cdot f_{\text{OFF}}) \cdot t)\) (Fig. 3c; \(P_{\text{ON}}\) = probability of OFF → ON transitions per unit time per endothelial cell; Supplementary Note 1). To test this, we quantified time-dependent accumulation of LacZ in vWF–Cre–ROSA26R mouse heart and liver capillaries co-immunostained for LacZ and pan-endothelial CD31, from birth to adulthood (Fig. 3d, Supplementary Fig. 4j–u). In line with our predictions, findings point to a random noise-driven process, resulting in dynamic mosaic VWF expression.

In addition to ever-present fluctuations in the rates of gene transcription, protein translation and regulatory network activity\(^{14–20}\), intracellular noise can also arise from asymmetric partitioning of a cell’s contents during division\(^{39}\). If cell division rate plays an important role in driving VWF ON/OFF transitions, \(P_{\text{ON}}\) should be higher in the early postnatal period when the endothelium is rapidly proliferating. However, the log-linear fit on Fig. 3e points to constant \(P_{\text{ON}}\) throughout the time window of

### Figure 2 | Organ-specific mosaic heterogeneity of VWF in vivo

Double immunofluorescence for VWF (red) and CD31 (green) of tissue sections and en face aortae from adult male wild-type (WT) mice (a–d), and LacZ staining of tissue sections and en face aortae from adult male vWFLacZ/+, (vWF knockin or vWF-KI) mice (e–h) and vWF-Cre–ROSA26R (vWF-CRE) mice (i–l). (a,e,i) Heart sections with capillaries cut longitudinally. (b,f,j) The inner lining of an aorta that has been opened and laid flat under a coverslip. (c,g,k) Heart sections with capillaries and veins cut in cross section. (d,h,l) Liver sections with hepatocytes surrounded by sinusoids (arrows). (a–l) VWF- or LacZ-expressing cells are indicated by solid arrows; non-expressing cells are indicated empty arrows; and LacZ-expressing hepatocytes are indicated by an *. Right column (m–p) shows our proposed model of a bistable switch regulating VWF expression, dynamically toggling between ON and OFF in heart capillaries (m), frozen into one of two states in the aorta (n) or locked into a single ON or OFF state in heart veins and liver sinusoidal endothelial cells, respectively (o,p). (q–s) Whole-mount LacZ staining of heart, muscle (diaphragm) and brain from adult male vWFLacZ/+(vWF-KI) mice and vWF–Cre–ROSA26R (vWF–CRE) mice. Scale bars, (a–l) 100 μm; (q–s) 3 mm, \(n=3\) with three replicates.

| vWF | LacZ | Switch |
|-----|------|--------|
| WT  | vWF-KI | vWF-CRE|
| Heart MVEC | Heart MVEC | Heart MVEC |
| Aortic EC | Aortic EC | Aortic EC |
| Liver MVEC | Liver MVEC | Liver MVEC |

| Heart | Diaphragm | Brain |
|-------|-----------|-------|
| vWF-KI | vWF-CRE | vWF-KI |
| vWF-CRE | vWF-CRE | vWF-CRE |
observation. Consistent with these observations, LacZ saturation in the adult vWF-CreERT2-ROSA26R mouse occurred on a similar time scale to that observed in young vWF-Cre-ROSA26R mice (Fig. 3b). These findings argue against a significant contribution of cell division to noise-induced VWF ON/OFF transitions.

**Figure 3 | VWF ON/OFF transitions occur in adult endothelium.** (a) Whole-mount LacZ staining of vWF-Cre-ROSA26R (vWF-CRE) hearts and diaphragms collected at indicated time points following birth (stained together and imaged side by side). Scale bar, 5 mm. (b) Time course of whole-mount diaphragm LacZ staining from tamoxifen-treated vWF-CreERT2-ROSA26R (vWF-CRE/TMX) mice. Time 0 (T0) indicates no exposure to tamoxifen. The results reveal time-dependent accumulation of LacZ in the capillaries, which are arranged in linear arrays parallel to the plain of the tissue. Scale bar, 2.5 mm. (c) Population dynamics of LacZ-expressing cell accumulation in vWF-Cre-ROSA26R endothelial cells (ECs), modelled by assuming random OFF → ON transitions with constant, division-independent rate \( P_{ON} \), time-dependent cell division with rate \( P_{D} \) and cell loss with rate \( P_{L} \). As indicated, the fraction of LacZ-negative cells, \( f_{OFF} \), decreases exponentially with time (see Supplementary Note 1 for further detail). Teal circle: LacZ-positive cell; white circle: LacZ-negative cell; circle with X: dead cell; black arrow: no promoter transition; blue arrow: OFF → ON transition of the endogenous promoter; grey arrow: ON → OFF transition of the endogenous promoter (masked by locked-in LacZ expression). (d) Left, Time-dependent LacZ accumulation in vWF-Cre-ROSA26R (vWF-CRE) mouse heart and liver capillaries (LacZ/CD31 double stained sections); Right, LacZ-positive EC percentage in heart and liver capillaries of 4-week-old vWF \(^{1/CRE}\)/LacZ mice (knockin, KI). (e) Log-linear plot of the LacZ-negative EC fraction, fitted by an exponential with rate \( P_{ON} = 0.02 \) per day per EC for heart, and \( P_{ON} = 0 \) per day per EC for liver. \( n = 3 \) with three replicates.

**Dynamic VWF mosaicism is driven by biological noise in vitro.** Stochastic transitions between VWF ON and OFF states may arise from random perturbations in the cell’s extracellular environment or from the stochastic variation in intracellular processes. To distinguish between these two possibilities, we turned to cell culture where we could more precisely control the extracellular environment. We employed fluorescence *in situ* hybridization (FISH) and immunofluorescence assays to evaluate VWF expression in monolayers of endothelial cells. Similar to our *in vivo* observations, and in contrast to the prevailing dogma that VWF is a universal marker for cultured endothelial cells, we found that VWF expression follows a mosaic pattern in multiple subtypes of primary human endothelial cells (Fig. 4a,b; Supplementary Figs 5 and 6a,b). Flow cytometric analysis of VWF protein and single-cell assays for VWF mRNA expression revealed a bimodal distribution in protein and mRNA, respectively. Individual VWF mRNA distributions normalized to VE-cadherin are well described by linear combinations of two binomial distributions with cell-type-specific mixing weights (Fig. 4c; fitting detailed in Supplementary Note 2). These findings suggest that VWF expression in cultured endothelial cells is mediated by a bistable ON/OFF switch, resulting in two distinct cell states. To prove that the VWF switch is dynamically regulated, we used limiting dilution to clone single
endothelial cells and then expanded each clone to \( \sim 10^6 \) cells. All clonally expanded populations demonstrated a mix of VWF low- and high-expressing endothelial cells, with VWF mRNA and protein distributions similar to those of the parent population (Fig. 4d–f). It is noteworthy that under \( \textit{in vitro} \) conditions, even human aortic endothelial cells demonstrated dynamic mosaicism, in contrast to the robust static mosaic pattern observed in mouse aortas \( \textit{in vivo} \). Together, these findings suggest that biological noise results in stochastic transitions between the ON and OFF states of a bistable VWF switch (alternative mechanisms inconsistent with our data are discussed in Supplementary Note 3).
DNA methylation correlates inversely with VWF expression. Since the in vivo rate of switching is on the order of weeks, we posited that the tight OFF state is maintained by epigenetic modification of the promoter. DNA methylation of promoter-region CpG dinucleotides is a potent epigenetic mechanism for silencing transcription. The human VWF promoter contains eight CpGs between nucleotide positions −900 and +400, relative to the transcriptional start site (Fig. 5a). We hypothesized that the methylation of one or more of these sites is an important determinant of the ON/OFF state of the gene. To test this, we carried sodium bisulfite genomic sequencing in several endothelial and non-endothelial cell types. This technique determines the methylation status of the eight CpG sites in the VWF promoter at the level of a single allele. For each cell type, we carried out bisulfite sequencing on at least 15 random VWF alleles. As shown in Fig. 5a, the VWF promoter was lightly methylated in multiple types of human endothelial cells (11–28% of the eight CpG sites), but heavily methylated in VWF-non-expressing cells, including vascular smooth muscle cells and hepatocytes (59–81% of the eight CpG sites). Overall, VWF expression correlated inversely with the average methylation of the eight CpGs (Fig. 5b; see Supplementary Fig. 7a and Note 4 for methylation sampling error estimates). Similar findings were observed with the mouse Vwf promoter (Fig. 5c). In agreement with the expected link between DNA methylation and chromatin configuration, we found a positive correlation between VWF expression levels and a transcriptionally permissive chromatin structure (Supplementary Fig. 7b–d). These findings suggest that both promoter DNA methylation and chromatin mechanisms contribute to differential expression of VWF across various cell types.

To determine whether differences in DNA methylation are also involved in mediating mosaic expression of VWF within a population of isogenic cells, we employed flow-activated cell sorting (FACS) to isolate single-donor human umbilical vein endothelial cells (HUVEC) with the highest (top 10%) and lowest (bottom 10%) levels of VWF and compared their DNA methylation using bisulfite genomic sequencing. As shown in Fig. 6a (left), the VWF promoter was significantly hypomethylated in the VWF-high subpopulation, compared with the VWF-low fraction. Similar results were observed with human aortic endothelial cells (Fig. 6a, right). To provide a more direct link between DNA methylation and VWF transcription, we used antibodies against RNA polymerase II to immunoprecipitate...
Figure 6 | DNA methylation is necessary and sufficient for silencing VWF expression. (a) Top, Bisulfite sequencing of the human VWF promoter in endothelial cells (ECs) that were FACs-sorted into subpopulations that expressed low levels of VWF (low) and cells that expressed high levels of VWF (high). Bottom, FACs profiles of VWF expression (left) and IgG control (right). The vertical red and black lines indicate the gating thresholds used for sorting. (b) Pol II ChIP assay followed by bisulfite sequencing of Pol II-bound and -unbound chromatin in human umbilical vein ECs (HUVEC) and human aortic ECs (HAoEC). (c) Repressive histone H3 lysine 9 dimethylation (H3K9Me2) ChIP assay followed by bisulfite sequencing of H3K9Me2-bound chromatin in HUVEC. Here, percentages denote the fraction of methylated CpGs at the first two CpG sites; dashes indicate unsequenced CpG sites due to lack of H3K9Me2 pull-down. (d) VWF mRNA expression and VWF promoter methylation following 5-azacytidine (5-Aza) treatment in HUVEC and HAoVSMC. VWF mRNA expression levels are shown relative to untreated vehicle controls. (e) Luciferase assay for methylated versus unmethylated VWF promoter/reporter constructs. Left: generation of 5′ VWF promoter/reporter constructs with mutations (CG→TG) at indicated CpG sites (●/○: non-methylated/methylated CpG sites); VWF promoter/reporter constructs were in vitro methylated using SsII methylase. Right: Luciferase activity in HUVEC transiently transfected with mock-methylated (white) or methylated (black) VWF constructs or pGL3 basic (units normalized to pGL3). HAoVSMC, human aortic vascular smooth muscle cells; Error bars indicate s.d.; n = 3, with three replicates. Two-sided T-test was performed in all significance tests. *P<0.05; **P<0.01; ***P<0.001.

transcriptionally active chromatin fragments in endothelial cells, and then carried out bisulfite sequencing on the Pol II-bound and -unbound chromatin fractions. These experiments showed that the Pol II-bound VWF alleles were consistently and significantly less methylated than the transcriptionally inactive Pol II-unbound VWF alleles (Fig. 6b). Moreover, DNA hypermethylated VWF alleles were associated with repressive histone H3 lysine 9 dimethylation (Fig. 6c).

To determine a causal link between VWF expression and DNA methylation, we carried out two additional assays. First, we measured VWF expression in various cells in the absence or presence of the DNA methyltransferase inhibitor 5-azacytidine (5-Aza). As shown in Fig. 6d, 5-Aza partially demethylated the VWF promoter, resulting in a seven- to eightfold increase in VWF mRNA expression in endothelial cells and de novo expression in otherwise VWF-negative vascular smooth muscle cells. Second, we used M.SssI to methylate CpG sites in the human VWF promoter (coupled to the lacZ reporter gene) and transfected the resulting episomal plasmid into human endothelial cells. Compared with the mock-methylated control, the in vitro M.SssI-methylated plasmid demonstrated significantly less promoter activity (Fig. 6e). Similar results were observed when only the proximal (Met 4) or the distal (Met 1) CpG sites were methylated. Taken together, these findings suggest that DNA hypermethylation of the VWF promoter is a repressive mark that is both necessary and sufficient for the VWF OFF state. Conversely, hypomethylation of the VWF promoter is required for the VWF ON state.
VWF DNA methylation spontaneously toggles ON and OFF. The above results raise the interesting possibility that dynamic regulation of DNA methylation is required for the noise-induced transitions between VWF ON and OFF states. To test this hypothesis, we performed bisulfite sequencing on clonal populations derived from single endothelial cells. In these experiments, we observed a mix of more than two methylation patterns in each assayed clone (Fig. 7a), indicating that the original pattern of DNA methylation of the two parental VWF alleles was not faithfully maintained (the likelihood of observing these CpG patterns due to experimental error is estimated in Supplementary Note 5 and Fig. 7e). Moreover, average VWF methylation was inversely correlated with VWF expression across independently derived clones (Fig. 7b). Overlay of these data onto the exponential fit from Fig. 5b, representing the methylation–expression relationship seen in parent endothelial cells and non-endothelial cells, demonstrated a similar relationship to that observed in parent cell lines (Fig. 7b). To directly prove the occurrence of ongoing VWF DNA methylation/demethylation, we performed hairpin bisulfite sequencing on three CpG sites in the core VWF promoter (Fig. 7c, top). This technique uses a short hairpin to link the top and bottom complementary strands of a double-stranded DNA near a region of interest, allowing for determination of the methylation state of a C nucleotide on both sides of a single CpG/CpG dyad. Any mismatch between methylation states of the two CpGs in a dyad is indicative of ongoing DNA methylation/demethylation. As shown in Fig. 7c, the methylation status was relatively stable.
in cells at the highest and lowest ends of VWF expression, namely in HUVEC, which demonstrate high-average VWF expression and low/absent methylation at the core promoter, and in human coronary artery vascular smooth muscle cells (HCVSMC) in which VWF is transcriptionally silent and heavily methylated. In contrast, we observed a high incidence of hemimethylated dyads in human cardiac microvascular endothelial cells (HMVEC) and human pulmonary artery endothelial cells (HPAEC), which express medium levels of VWF. Following the stochastic model of DNA methylation proposed in ref. 47, we used these data to estimate the rates of methylation maintenance ($E_{\text{in}}$), demethylation ($1 - E_{\text{in}}$) and de novo methylation ($E_{\text{out}}$) in different cell types (Supplementary Note 6). Endothelial cells with a high incidence of hemimethylated CpG dyads demonstrated higher rates of DNA methylation loss (failure of maintenance and/or increased demethylation; 0.56 per CpG per division in HPAEC and 0.71 per CpG per division in HMVEC) compared with HCVSMC (0.1 per CpG per division; Fig. 7c). Together, these findings suggest that both VWF expression and CpG methylation flickers ON and OFF in isogenic cells.

We next asked whether DNA methylation constitutes the binary switch itself48,49 (please see Supplementary Note 7 for an explanation of how DNA methylation may theoretically create such a switch), or whether it simply ‘marches to the tune’ of an upstream noise-sensitive bistable switch. We reasoned that if DNA methylation (with associated changes in chromatin configuration) is sufficient for the switch-like behaviour of VWF, then the two Vwf alleles should toggle independently. If, on the other hand, the switch is located in an upstream regulatory pathway, the two alleles should be co-regulated. To test this, we isolated cardiac microvascular endothelial cells from VWF$^+/+$ mice (in which one Vwf allele drives VWF expression, and the other drives LacZ expression) and carried out co-FISH staining for VWF and LacZ (Fig. 8a). As shown in Fig. 8b, there was a strong correlation between VWF and lacZ mRNA numbers in individual cells, arguing against independent switching of the two alleles and in accord with previous reports for other loci50,51. Instead, the coupled activity of the two alleles suggests that the relevant noise source is upstream of the Vwf promoter, and that dynamic VWF mosaicism is driven by a bistable regulatory network that converges on the transcriptional and/or DNA methylation machinery at the Vwf promoter (Fig. 8c).

**VWF expression is required for cardiac health.** An important question is whether stochastic transitioning between VWF ON/OFF states is functionally relevant. To address this, we focused on the adult heart. We reasoned that if mosaic VWF expression in the heart has an adaptive role, then loss of expression from the minority of normally VWF-positive cells in VWF$^-$ mouse hearts is responsible for this effect.

Next asked what mechanism might be responsible for the deleterious effect of VWF knockout on cardiac health. Based on the well-recognized role of VWF in mediating platelet adhesion to the subendothelial surface of injured blood vessels5-52, we anticipated that the cardiac phenotype in VWF-null mice would be attributed to focal microhemorrhaging in the capillary bed. However, we could not detect any evidence of red blood cell extravasation or plasma leak in the heart. Interestingly, recent studies have implicated non-haemostatic roles for VWF53-55, including the modulation of angiopoietin-Tie signalling56. We present data suggesting that dynamic mosaicism of VWF expression in the capillaries of the heart is associated with randomly shifting hotspots of sustained Ang2 secretion, and that disruption of the mosaic leads to dysregulated Ang2 release and secondary microvascular damage (Supplementary Note 9 and Fig. 10).

**Dynamic mosaicism is not limited to VWF.** To determine whether the dynamic mosaic heterogeneity observed with VWF is limited to that gene or represents a more generalized phenomenon in endothelial cells, we carried out FISH analysis of four different endothelial-restricted genes in parent and clonal populations of cultured human endothelial cells: endothelial-specific molecule 1 (ESM1, important for sprouting angiogenesis57); ephrin-B2 (ref. 58) and ROBO4 (ref. 59) (involved in vascular patterning); and the endothelial-specific transcription factor ERG60. These experiments revealed mosaic mRNA expression of ESM1 and ephrin-B2 in parental and clonal populations of isogenic cells, and uniform expression of ROBO4.
and ERG (Fig. 9). These findings suggest that dynamic mosaic heterogeneity is not unique to VWF, and may represent a more widespread adaptive behaviour of the endothelium.

Discussion
Mechanisms of endothelial heterogeneity are often attributed to differences in the extracellular environment (‘nurture’) or to memory of past differences encoded by epigenetic modifications (‘nature’). However, a different mechanistic framework is required to explain dynamic mosaicism, in which endothelial phenotypes spontaneously flip in adjacent cells exposed to near-identical microenvironments. To this end, we turned to dynamical systems theory to reframe endothelial cell heterogeneity in terms of multistability, the ability of single cells to maintain distinct phenotypes under identical extracellular conditions (the ‘nature’ side of the equation). Multistability is generated through positive regulatory feedback and/or epigenetic modifications that generate barriers between distinct states. These states may be represented as valleys on a landscape, with the internal state of an endothelial cell represented as a marble on its surface (Fig. 1). Endothelial cells constantly navigate this landscape in response to extracellular signals, as well as intracellular noise. When exposed to appropriate signals, cells may cross barriers and enter other valleys (the ‘nurture’ side of the ledger). Biological noise results in pervasive cell state fluctuations within a valley. When barriers are sufficiently low, as in the case of VWF in capillary endothelial cells of heart, skeletal muscle, lung and brain, noise leads to stochastic transitions between two distinct phenotypes. Consequently, the mosaic pattern ‘blinks’ ON/OFF during the lifetime of a cell (Fig. 10). Correlated expression of the two VWF alleles (Fig. 7a,b) suggests that stochastic ON/OFF transitions of the promoter are mediated not by stochastic interactions between RNA polymerases and DNA (that is, intrinsic noise), but rather by upstream (i.e., extrinsic) noise that converges on both alleles.

Since extrinsic noise typically affects the expression of several genes, it is likely that the vWF ON/OFF states are associated with distinct transcriptional profiles. In future studies, it will be interesting to identify the origin of the extrinsic noise responsible for toggling vWF expression, and to identify those genes that are co-regulated with vWF.

Biological noise provides a source of cell-type diversification in clonal populations, and can confer a fitness advantage to cell collectives or an organism. In bet hedging, unicellular organisms stochastically commit some fraction of their population to a
VWF predisposes to a bleeding phenotype, whereas excessive—or in capillaries of the heart. VWF is a haemostatic protein that VWF-null mice have a markedly abnormal endothelial phenotype. A final explanation, which we favour, is that VWF switching itself co-regulation of another gene that provides a selective advantage. Stochastic transitions in VWF expression may be a byproduct of stochastic decision-making, the new state (ON or OFF) has gone irreversible and is not the dynamic toggling between ON and OFF states. This question could only be addressed by designing a mouse in which VWF expression in the heart is converted from a dynamic to a static mosaic.

These various limitations notwithstanding, our data are the first to support the existence of noise-induced stochastic phenotype switching in adult mammals. Our findings point to biological noise as a new source of phenotypic diversity in the healthy endothelium, which in the case of VWF is exploited by some, but not all, vascular beds. The observation that other genes, including ESM1 and ephrin-B2, also demonstrate dynamic mosaicism in vitro raises the possibility that this phenomenon is not unique to VWF but may represent a general, previously unappreciated endothelial cell behaviour.

The traditional motivation for studying both the scope and the molecular basis of endothelial heterogeneity is to design vascular bed-specific therapies. The implicit goal is to inhibit (that is, turn OFF) or accentuate (that is, turn ON) site-specific expression/activity of certain proteins to modulate local endothelial cell behaviour. However, in so far as noise-induced transitions in phenotype are adaptive, therapies aimed towards nudging cells into a single state may be ill advised. The optimal treatment may be one that restores the flexibility to generate mosaic heterogeneity, and thus preserve homoeostatic bet hedging.

**Methods**

**Statistics.** Two-sided $T$-test was performed in all significance tests, except the clonality assessment of DNA methylation patterns (Supplementary Note 7).

**Approval for experiments involving animals.** All experiments involving the generation, manipulation and analysis of mice were carried out in compliance with ethical regulations and were approved by the Institutional Animal Care and Use Committee at the Beth Israel Deaconess Medical Center (BIDMC).
Generation of targeting constructs. To generate the VWFLaCZ– construct, RPCI-24 mouse genomic BAC clones 365D10 and 211J14 (in Erichsichia coli EL250 cells were grown under chloramphenicol selection, heart-shocked to induce recombination activities and electroporated with the LacZ-pA-fritted neo cassette from pB2XZeoB with ligated homology arms to direct its insertion between the end of intron 1 and nucleotide 4253 of intron 3. The scheme used to capture the native locus in the construct was pVWMDT2Z- to capture this targeted locus. To generate the VWFCreC– construct (pVWKOCKI), recipient proficient cells were co-electroporated with pVWMDT2Z and a Cre-pA-fritted neo cassette with ligated homology arms to direct its insertion between the end of exon 1 and nucleotide 4253 of intron 3. The cassette was constructed in three steps. First, a pair of complementary oligos was inserted between blunt-ended SpI and BamHI sites of pUC18 to introduce a flippase recognition target (FRT) site preceding by sites for BbsI and PmlI and followed by another BbsI site oriented opposite the first. The PmlI site and the XbaI site in FRT are unique. Next, a nuclear-localized Cre recombinase coding sequence and human β-actin polyadenylation sequence amplified from pML78 was inserted into the flanking FRT sites. The orientation of Cre recombinase in the preceding step was chosen to be parallel to neo resistance in the ultimate product. Digestion of this construct with BbsI (after exhaustive HindIII digestion to linearize it) released the Cre-pA-fritted neo cassette as two fragments due to an overlooked internal polyadenylation sequence amplified from pML78 was inserted into the PmlI site. Finally, an XbaI restriction production following the neo resistance cassette of pNEOREV was inserted into the XbaI site in the orientation that preserved the flanking FRT sites. The orientation of Cre recombinase in the preceding step was chosen to be parallel to neo resistance in the ultimate product. Digestion of this construct with BbsI (after exhaustive HindIII digestion to linearize it) released the Cre-pA-fritted neo cassette as two fragments due to an overlooked internal BbsI site. Each homology arm was amplified with one primer that introduced a BamHI site and sequence that on digestion complemented the appropriate BbsI digested end. The homology arms and the two cassette fragments were ligated and employed in the above recombination reaction. To generate the VWFCreERT2– construct, targeting construct containing full-length Cre-pA-fritted neo cassette with ligated homology arms to direct its insertion between the end of exon 1 and nucleotide 4253 of intron 3. Construction of the cassette employed the initial preparation from construction of the pVWKOCKI cassette, but in this case the next step was insertion of the neo cassette from pNEOREV to pVWKOCKI. 2,348-bp amplicon from pCAG-CreERT2 (Addgene ID 14797) containing coding sequence and polyadenylation signal was inserted into the PmlI site. Digestion of this construct with BbsI (after exhaustive EcoRI digestion to linearize it) released the inducible Cre-pA-fritted neo cassette. This was ligated with the same homology arms used in the construction of pVWKOCKI.

Generation of gene-targeted mice. To generate VWFLaCZ, VWFCreC– and VWFCreERT2– mice, W41289 embryonic stem cells (ES cells) (Taconic Biotechnology) were prepared and transfected with the constructs described above, placed under G418 selection (Invitrogen) for 6–7 days. Homologous recombination in G418-resistant ES cells was confirmed by Southern blot of genomic DNA and specifically designed probes. Positive clones were amplified and submitted to the BIDMC transgenic facility for injection into blastocytos and TiceCre+/+ offspring were obtained from Jackson Research Laboratories. To generate VWFCreERT2–ROSA26, mTie2–Cre–ROSA26, or Tie2–Cre–ROSA26 mice, VWFCre/– mice, VWFCre+– mice, and TiceCre+– mice were crossed with ROSA26 mice (Jackson Research Laboratories). Each of these lines has been backcrossed to C57BL/6 for at least nine generations. Unless otherwise stated, all experiments involved male mice between the ages of 6 and 8 weeks.

LacZ staining and VWF immunohistochemistry. Tissues were collected and fixed in fixative solution containing 270 µl of formaldehyde and 80 µl of glacial acetic acid in 10 ml PBS for 30 min at room temperature. The tissues were washed three times in PBS, and then either incubated with X-gal overnight for whole-mount staining, or embedded in optimal cutting temperature compound (OCT), sectioned, fixed a second time with fixative solution for 10 min and incubated overnight with X-gal. For immunohistochemistry, slides were fixed in acetone at −20 °C for 2 min, rinsed three times in PBS, and incubated in Dako Ready-to-use Protein Block (Dako Cat. No. X0909) for 10 min at room temperature. Slides were then incubated with primary antibody (polyclonal anti-human VWF, Dako Cat. No. A0082) 1:100 in PBS for 4 h at room temperature. The slides were washed three times in PBS, blocked with peroxidase (1 ml H2O2 in 150 ml methanol) for 10 min at room temperature and washed again before incubating with secondary antibody (biotinylated goat anti-rabbit, Dako Cat. No. E0432). After washing in PBS, the slides were incubated with tertiary antibody (Vector Labs ABC Elite PK-6100) for 45 min at room temperature. Finally, the slides were stained with HemaToxylin and saturated Lithium Carbonate Solution and sealed with cover slips in aqueous mounting medium.

Immunofluorescent co-staining of tissue sections. Frozen sections of organs were fixed in cold acetone for 5 min at −20 °C, rinsed in PBS and blocked in blocking solution (3% fish gelatin and 10% donkey and 10% goat sera) for 1 h at room temperature and then incubated with primary antibodies (chicken polyclonal anti-LacZ, Abcam ab9631) diluted 1:500 in GDMM supplemented with 10% FCS (Hyclone). All primary human endothelial cells were obtained from Lonza and grown in EBM-2 (EC Basal Medium-2) supplemented with EGVM-MV SingleQuots (Lonza). HCSVSMC and HAoVSMC were also obtained from Lonza and grown in SmBM Basal Medium supplemented with SmGM-2 SingleQuot (Lonza). To isolate primary mouse lung and heart endothelial cells, mouse tissue was harvested on ice, minced and digested in 0.2% (w/v) type 1 collagenase (Collagenase) for 45 min at 37 °C, passed over a 70 micron filter, and spun down at 500G without brake (4 °C). The resulting cell pellet was resuspended in bead wash (PBS + 0.1% albumin and antibiotics) and then incubated for 15 min at room temperature with magnetic beads (Dynabeads, Invitrogen) that were precoated with anti-mouse CD31 antibody (BD Biosciences, Cat. No. 553370). After five washes, the cells were harvested for T0 assays, or plated on gelatin-treated tissue culture plates (0.1% gelatin, Millipore) and grown to confluence in DMEM with 20% FCS, amino acids, glutamax, antibiotics and B27/20 endothelial mitogen (Bioscience Technology, Inc.). A second beading was performed with anti-rat IgG (Invitrogen) conjugated with magnetic beads coated with anti-mouse ICAM2 antibody (BD Biosciences, Cat. No. 553326) and plated on gelatin-treated plates. Primary mouse hepatocytes were purchased from BD Biosciences. To obtain mouse sinusoidal endothelial cells, freshly harvested livers were washed with Dulbecco’s PBS (DPBS) and minced. Liver material was then suspended in 0.2% (w/v) type 1 collagenase (Worthington) in DPBS and incubated in an overhead shaker at 37 °C for 45 min. Liver cells were further dissociated by repeated suction through a 14-gauge needle and filtered through a 70-µm cell strainer (BD Biosciences). The cell suspension was centrifuged for 1 min at 500G without brake (4 °C). The HepG2 containing pellet was washed and resuspended in DPBS and incubated with OptiPrep (Nycomed, Invitrogen) that was diluted at the ratio of 3:1, layered with DPBS and centrifuged for 15 min at 1500G without brake (4 °C). Cells in the interphase were collected, washed in DPBS, centrifuged for 10 min at 500G and resuspended in bead wash buffer (PBS with antibiotic-antimycotics and BSA; all from Mediatech, Herdon, VA). Next, Dynabeads sheep anti-rat IgG (Invitrogen) conjugated with magnetic beads CD31 monoclonal antibody (BD Biosciences Cat. No. 558736) were added and the mixture was incubated in an overhead shaker for 15 min at room temperature. Cells were repeatedly washed with bead wash buffer using a magnetic cell sorter before harvesting in DNA isolation buffer.

Fluorescent in situ hybridization. FISH was performed using QuantGene ViewRNA ISH Cell Assay Kit (Affymetrix), following the manufacturer’s instructions with minor modifications. Briefly, cells were plated on coverslip coated coverslips, fixed in 4% formaldehyde for 5 min, permeabilized with detergent solution for 5 min and treated with protex for 10 min at room temperature and incubated with gene-specific probes for 3 h at 40 °C (probes, hVWF: nmo_000525, 2460–3950; hESMi: nm_007036, 542–2049; hRHOBO4: nmo_019055, 713–1896; hVE-CAD: nm_001795, 284–1406; hHFN2B: nmo_004093, 545–799; hEGR: nm_004449, 107–1179; nmVH: nm_011708, 3702–4782. LacZ, U64849, 519–1433), followed by incubation with preamplifier Mix, Amplifier Mix and Label probe Mix, sequentially. The slides were rinsed three times in PBS and mounted with coverslips using VectaShield mounting medium containing 4,6-diamidino-2-phenylindole for nuclear imaging. Images were collected using Zeiss LSM 510 laser-scanning confocal microscope with a 40 x oil-immersion objective.

Quantitative real-time PCR. Total RNA was isolated using the RNeasy kit (Qiagen). Single-stranded complementary DNA (cDNA) was synthesized from total RNA using the RNA-to-cDNA kit (Applied Biosystems). SYBR Green 1-based detection was used in a 96-well plate with an ABI7500 Real Time PCR instrument. cDNA, 18S ribosomal RNA were used to normalize the amount of cDNA. The sequences of the primers used in this study are listed in Supplementary Table 1.
Arterial and venous flow experiments. HPAEC were plated onto 0.1% gelatin-coated glass coverslips at a cell density of 72,000 cm⁻². The samples were exposed for 24 h to a beaker containing stress wash and carbon dioxide in the absence of any oxygen or glucose. After 24 h, the culture medium was replaced with fresh medium, and the samples were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO₂ to yield individual strands, followed by sequencing. For each cell type at least 15 randomly chosen sublines were sequenced. Primers used for human VWF upstream region were hvWF-upstream-forward-2, hvWF-upstream-forward-3, hvWF-upstream-forward-4, hvWF-upstream-forward-5, hvWF-upstream-forward-6, hvWF-upstream-forward-7, hvWF-upstream-forward-8, hvWF-upstream-forward-9, hvWF-upstream-forward-10, hvWF-upstream-forward-11, hvWF-upstream-forward-12, and hvWF-upstream-forward-13. Primers used for mouse VWF proximal promoter region were mVWF-prox-forward-2, mVWF-prox-forward-3, mVWF-prox-forward-4, mVWF-prox-forward-5, and mVWF-prox-forward-6. These sequences of these primers are listed in Supplementary Table 1. The Chinese Han Genome (CHB) Bisulfite Sequencing (BS-seq) panel was designed to cover all regulatory regions analysed by the bisulfite sequencing method. The CHB panel was validated using DNA samples from 14 healthy individuals of Han Chinese ancestry. The performance of the CHB panel was compared to a previously published panel [16].

Correlation between average mRNA level and DNA methylation. To quantitate the relationship between average DNA methylation and mRNA expression, we used a log-linear fit on all assayed cell types and endothelial cell subtypes (that is, log(mRNA)~average DNA methylation). Cell types used for plot: HUVEC, HAEC—two separate preparations, venous flow, arterial flow; HMVEC—two separate preparations; human aortic endothelial cells, HCAVMSCs and hepatocytes (all were primary human cells). The resulting exponential, shown in Fig. 5b, is described by $y = y_{0} - b \times e^{-c \cdot x}$, with a correlation coefficient of $C = 0.95$.

CHBP and bisulfite sequencing of CHB DNA. Chromatin immunoprecipitation (ChIP) was performed on post-confluent primary cells using the CHB Assay Kit (Millipore) according to the manufacturer’s protocol. Sonication yielded soluble chromatin fragments containing DNA ranging in size from 200 to 400 bp. Histone H2A (C terminus) rabbit polyclonal antiserum (GeneTex, Cat. No. GT122), histone H4 rabbit polyclonal antiserum (GeneTex, Cat. No. GT12), histone H3 rabbit polyclonal antiserum (Abcam, Cat. No. ab8493), and histone H3 acetylated (H3ac) rabbit polyclonal antibody (Abcam, Cat. No. ab5699). All other reagents were purchased as described in Supplementary Table 1.

5-Aza treatment of cultured cells. HUVECs and HAoVSMC were seeded at a density of 5 x 10^6 cells on 100 mm dishes. Exponentially growing cells were treated with 5-Aza (Sigma-Aldrich) every 24 h for 7 days. Maximal tolerable dose of 5-Aza was used for HUVECs (0.25 μM) and HAoVSMC (0.5 μM). Medium was changed on the fourth and sixth days of treatment. On the eighth day, cells were harvested for genomic DNA and total cellular RNA.

Hairpin bisulfite sequencing. Given that the nascent strand of replicating DNA is hemimethylated immediately following DNA replication, only quiescent post-confluent primary cells were utilized for DNA isolation. Briefly, 2 μg of mechanically sheared genomic DNA was denatured with 0.3 M NaOH for 15 min at 37°C, then hybridized with 3.1 M sodium bisulfite and 0.5 M m-hydroquinone at 55°C for 16 h in the absence of light. Free bisulfite was removed using UltraClean 15 DNA Purification kit (MO BIO Laboratories). The labelled cells were washed with PBS, Flow cytometry runs and FACS sorting were performed on the FACSCalibur D04344 and FACScalibur FACSorter FAC, using the CXP analysis software. For FACS sorting, the highest and lowest 10% were washed with PBS. Flow cytometry runs and FACS sorting were performed on the FACSCalibur D04344 and FACScalibur FACSorter FAC, using the CXP analysis software. For FACS sorting, the highest and lowest 10% were washed with PBS.
but with the addition of a heat-denaturing step (99°C for 2 min) during the 90 min of bisulfite conversion (65°C) to denature the hairpin-linked DNA. Bisulfite-treated DNA was subjected to 40 cycles of PCR amplification in a volume of 50 μl using VWF-specific forward primer hVWF-hairpin-F and reverse primer hVWF-hairpin-R; annealing temperature was 47.5°C. 10 μl of the PCR product (586 bp) was used as template for another 40 cycles of nested PCR amplification in a volume of 50 μl using VWF nested forward primer hVWF-hairpin-nested-F and reverse primer hVWF-hairpin-nested-R; annealing temperature was 46.4°C. The final PCR product (550 bp), containing CpG dyads at +119, +179 and +227 of human VWF, was subcloned using TOPO TA cloning kit to yield individual DNA molecules, followed by sequencing. Randomly variable barcode encoded in the loop of the hairpin linker was used to identify and remove redundant methylation patterns arising from PCR amplification of limited amounts of DNA template.⁶⁶

**Transmission electron microscopy.** Plastic sections (1 μm) were stained with alkaline Giemsa stain for study by light microscopy, and 70- to 80-nm thin sections were examined by electron microscopy with Philips 300, 400 and CM10 electron microscopes (Philips, Eindhoven, The Netherlands) by three different microscopists.

**PV loop, echocardiography and aortic banding.** For pressure–volume loop, pressure–volume parameters were measured under isoflurane (2%) inhalant anesthesia using a 1.4 Fr microtip pressure–volume catheter (Sciensc), inserted into the right common carotid artery and advanced into the left ventricle. Data were recorded on a PowerLab system (AD Instruments). Beat-by-beat pressure–volume parameters including stroke work, cardiac output, preload, afterload and contractility were measured and analysed using CardioSoft Pro software (CardioSoft). For echocardiography, the chest hair was removed and two-dimensional images were visualized using a Vevo 2100 system. Non-invasive M-mode imaging could accurately measure cardiac function, left ventricular wall thickness and chamber dimensions. For surgical aortic banding, mice under anesthesia were placed supine on a heating pad. A horizontal skin incision of 0.5–1.0 cm in length was made at the level of the suprasternal notch and aortic arch was accessed. Following ligation, the needle was quickly removed. The skin incision was then placed next to the aortic arch and the suture was tied around the aorta. Following ligation, the needle was quickly removed. The skin was closed and mice were allowed to recover. The sham procedure was identical except that the aorta was not ligated.

**Assaying liver and kidney function.** Blood was collected via cardiac puncture and assayed for liver and kidney function using Chem-27 assay commercial services provided by the Diagnost Lab in the Division of the Comparative Medicine, Massachusetts Institute of Technology.

**Angiopoietin 2 ELISA.** was performed using Mouse/Rat Angiopoietin 2 Quantikine ELISA Kit (antibody1:200; R & R&D Systems) following the manufacturer’s instruction.
Acknowledgements

We are grateful to Jan A.A.M. Kamps and Peter J. Zwierts for their technical help with harvesting liver endothelial cells, to Claudia Prahst for her technical help with β-galactosidase/CD31 co-staining of aorta preparations, to Dan Li and Shou-Ching Jainmet for their technical help with qPCR assays, to Joel Lawitts for generating mice, and to Chandra Gosh and Zoltán Toroczkai for their helpful discussions on the broad significance of our work. This work was supported by National Institutes of Health: National Heart, Lung and Blood Institute, grants HL119322 (W.C.A. and E.R.R.) and HL076540-10 (W.C.A. and G.G.-C.), as well as the American Heart Association: grant 14STG18590011 (L.Y.).

Author contributions

L.Y., G.C.C., E.R.R. and W.C.A. designed all the studies. L.Y. and G.C.C. performed most of the experiments. E.R.R. performed the simulations and modelling. D.B. designed and cloned the targeting constructs and luciferase constructs. K.S. generated and genotyped the targeted ES cells. L.J. maintained, bred and genotyped the mouse lines. L.Y. and H.D. carried out staining of mouse tissues and cultured endothelial cells. A.M. and N.J. carried out immunofluorescent studies of heart and liver tissues. W.J.A. and G.G.-C. conducted the flow experiments. G.M. provided DNA from liver sinusoidal endothelial cells from mice and provided valuable input on experimental design. P.A.M. provided DNA from various human endothelial cells and provided valuable input on experimental design. T.S. and A.D. performed the electron microscopy studies. L.Y. and P.K. performed echocardiography and aortic banding studies. L.Y., G.C.C., E.R.R. and W.C.A. wrote the paper.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Yuan, L. et al. A role of stochastic phenotype switching in generating mosaic endothelial cell heterogeneity. Nat. Commun. 7:10160 doi: 10.1038/ncomms10160 (2016).

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