Vasculopathy-associated hyperangiotensinemia mobilizes haematopoietic stem cells/progenitors through endothelial $\text{AT}_2\text{R}$ and cytoskeletal dysregulation

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Patients with organ failure of vascular origin have increased circulating haematopoietic stem cells and progenitors (HSC/P). Plasma levels of angiotensin II (Ang-II), are commonly increased in vasculopathies. Hyperangiotensinemia results in activation of a very distinct Ang-II receptor set, Rho family GTPase members, and actin in bone marrow endothelial cells (BMEC) and HSC/P, which results in decreased membrane integrin activation in both BMEC and HSC/P, and in HSC/P de-adhesion and mobilization. The Ang-II effect can be reversed pharmacologically and genetically by inhibiting Ang-II production or signalling through BMEC AT$_2$R, HSCP Ang-II receptor type 1 (AT$_1$R)/AT$_2$R or HSC/P RhoA, but not by interfering with other vascular tone mediators. Hyperangiotensinemia and high counts of circulating HSC/P seen in sickle cell disease (SCD) as a result of vascular damage, is significantly decreased by Ang-II inhibitors. Our data define for the first time the role of Ang-II HSC/P traffic regulation and redefine the haematopoietic consequences of anti-angiotensin therapy in SCD.
Hematopoietic stem cells and progenitor (HSC/P) are needed constantly to replace mature blood cells. The bone marrow (BM) microenvironment regulates a homeostatic dynamic equilibrium with a pool of circulating HSC/P. The size of the circulating pool of HSC/P is critically regulated by their adhesion or de-adhesion to their microenvironment. Patients with organ failure of vascular-endothelial origin have an increased circulating pool of HSC/P and which may represent a homeostatic stress response contributing to vascular damage repair. An example of systemic vascular-endothelial disease is sickle cell disease (SCD) which is associated with endothelial activation and damage and an increased pool of circulating primitive haematopoietic progenitors.

Angiotensin II (Ang-II) is the major bioactive peptide of the renin–angiotensin system and is implicated in the homeostatic regulation of blood volume, vascular tone and sodium retention. Patients with vascular disease commonly develop secondary hyperangiotensinemia and BM dysfunction of multifactorial origin. There is evidence for the effect of Ang-II in haematopoiesis. Studies have shown that animals and patients receiving Ang-II targeted therapies had decreased stress erythropoiesis, suggesting the existence of a local Ang-II regulatory system in the BM that is involved in the regulation of haematopoiesis. However, the intrinsic or extrinsic nature of the mechanisms by which Ang-II regulates HSC/P activity in pathological conditions remains unknown.

In this study, we reveal that acute and chronic hyperangiotensinemia in mice results in an increased pool of circulating HSC/P, which can be reversed pharmacologically or genetically by a microenvironmental deficiency of Ang-II receptor type 2 (AT2R). Hyperangiotensinemia results in HSC/P de-adhesion from BM endothelial cells (BMEC) through distinct changes in the balance of the activated Rho family GTPases, Rho and Rac, and in cytoskeletal rearrangements in the BMEC and HSC/P. We show that untreated patients with SCD have high levels of Ang-II and HSC/P in their peripheral blood, which significantly decreases on angiotensin-targeted pharmacological or genetic intervention. These results indicate a new role for angiotensin in HSC/P trafficking under pathological conditions, and define the haematopoietic consequences of anti-angiotensin therapy in SCD.

**Results**

**Hyperangiotensinemia increases the pool of circulating HSC/P.**

To understand whether hyperangiotensinemia results in increased mobilization of HSC/P, we first used a murine model of chronic hyperangiotensinemia secondary to a vasculopathy. An EC deficiency of Cx43, a gap junction forming protein that is crucial in maintaining EC-to-EC junctions and preserving the barrier function of EC, has been shown to result in chronic effective hypovolemia, hypotension and secondary hyperreninemic hyperangiotensinemia. We have generated independently endothelial Cx43-deficient mice by crossing Tie2-Cre expressing mice with biallelic Cx43 exon 2 floxed mice, demonstrated that these mice lack expression of Cx43 in BMEC, and recombinated the Cx43 gene in colonies derived from peripheral blood (PB) while still expressing Cx43 in the BM stromal cells (Supplementary Fig. 1a,b). We have named these mice HyperAng-II-Cx43-EC mice to summarize this dual property of deletion of Cx43 in EC and hyperangiotensinemia. HyperAng-II-Cx43-EC mice had normal numbers of PB leucocyte subpopulations (Supplementary Fig. 1c–f), blood haemoglobin, and erythrocyte and platelet counts (Supplementary Fig. 1g–i), but had a two to threefold increase in the number of circulating myeloid committed haematopoietic progenitors (Fig. 1a) and repopulating stem cells (Fig. 1b) compared with their wild type (WT) control littermates. Immunophenotypic enumeration of circulating HSC/P in HyperAng-II-Cx43-EC mice indicated that both the HSC and different populations of committed progenitors, including long-term HSC, short-term HSC, multipotential progenitors, common myeloid progenitors, granulomacrophagic progenitors and megakaryoblastic-erythroid progenitors, were consistently increased two to threefold (Supplementary Fig. 2a–e). The increase in circulating HSC/P accounts for ~0.1% competitive repopulating units (CRU) and ~0.5% colony forming units (CFU)-C, respectively, of all BM HSC/P. These levels are similar to the mobilization with the CXCR4 inhibitor, AMD3100 (ref. 21). As expected, the BM content of immunophenotypically defined HSC/P (Supplementary Fig. 2f–g) as well as functional progenitors (Fig. 1c) and competitive repopulating stem cells (Fig. 1d) were not significantly different in HyperAng-II-Cx43-EC mice compared with their WT control littermates, which explains the apparent absence of changes in the content of HSC/P in the BM. Similar to BM, there was no significant change in the splenic content of HSC/P in the HyperAng-II-Cx43-EC mice (Fig. 1e). Interestingly, the deficiency of Cx43 in HSC/P alone does not induce HSC/P mobilization confirming the existence of a non-cell-autonomous effect of Cx43 deficiency in HyperAng-II-Cx43-EC mice. Generation of chimeric animals with normal haematopoiesis and Tie2-Cre-Cx43-deficient microenvironment (Fig. 1f) phenocopied the increased level of circulating HSC/P, confirming that the increase in circulating HSC/P in HyperAng-II-Cx43-EC mice was of non-cell-autonomous origin (Fig. 1g). The increased number of circulating HSC/P in these mice was not associated with increased circulating levels of the chemokines Cxcl12 (Supplementary Fig. 2h) or stem cell factor (Supplementary Fig. 2i), which are expressed by BMEC and BM stromal cells, and are reported to function as major regulators of HSC/P content and trafficking. β-adrenergic stimulation has also been shown to be critical in controlling HSC/P egress. We did not observe any significant changes in the levels of norepinephrine or epinephrine in the BM (Supplementary Fig. 2j–k) or blood (Supplementary Fig. 2l–m) of HyperAng-II-Cx43-EC mice, nor did the β-adrenergic blocker propranolol have an effect on their circulating HSC/P counts (Supplementary Fig. 2n). To demonstrate whether the increased level of Ang-II was responsible for the increased circulation of HSC/P, HyperAng-II-Cx43-EC mice were given the angiotensin-converting enzyme (ACE) inhibitor enalapril, which blocks the transformation of Ang-I into Ang-II. Enalapril was effective in reducing the plasma levels of Ang-II in HyperAng-II-Cx43-EC mice and restored the increased count of circulating/mobilized HSC/P to levels similar to control animals (Fig. 1h–i), indicating that the ACE-mediated formation of Ang-II is implicated in the mobilization of HSC/P to the PB.

**Ang-II induces HSC/P mobilization.** We then analyzed whether the HSC/P mobilization required chronic exposure to high levels of Ang-II or whether it could be achieved by transient hyperangiotensinemia as a result of a bolus administration of Ang-II. WT mice were administered with 1.44 mg kg⁻¹ Ang-II dissolved in isotonic phosphate buffered saline (PBS) intraperitoneally, which resulted in increased plasma levels of Ang-II at 1, 5 and 15 min after administration, returning to basal levels by 30 min after infusion (Supplementary Fig. 2o). The administration of Ang-II resulted in significantly increased circulation of HSC/P in PB within 15 min post-infusion, which returned to basal levels by 30 min post-infusion (Fig. 2a). Furthermore, continuous infusion of Ang-II, reaching an approximately three fold increase of plasma Ang-II levels over the endogenous production...
Hydroangiotensinemia

Two distinct Ang-II receptor subtypes, AT1R and AT2R, which have been identified on the basis of their differential pharmacological and biochemical properties induce specific signalling pathways and seem to play distinct and sometimes opposite roles in vascular regulation. Both AT1R and AT2R are expressed by BMEC and HSC/P (Supplementary Fig. 3c). To elucidate whether AT1R or AT2R were specifically responsible for the HSC/P mobilization phenotype, we evaluated the level of HSC/P mobilization after Ang-II administration in models of loss-of-function of Ang-II receptors. AT1R−/− and AT2R−/− mice were given a single dose of Ang-II and the level of circulating HSC/P was analyzed. While the deficiency of AT1R did not modify the effect of Ang-II on HSC/P mobilization, the single deficiency of AT2R completely prevented the increased HSC/P mobilization response after pharmacological administration of Ang-II (Fig. 2c), or in our model of chronic hydroangiotensinemia with Ang-II deficiency (Fig. 2d), indicating that AT2R is indispensable to mediate the mobilization of HSC/P induced by Ang-II.

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To examine whether Ang-II/AT2R activity was cell autonomous or not, and whether the expression of other murine AT2R subtypes might have been overlooked in our genetic murine model of AT1R deficiency, we generated chimeric animals by transplanting BM HSC/P from congenic WT CD45.1+ mice into CD45.1+ mice (Supplementary Fig. 3c). To elucidate whether AT1R or AT2R were specifically responsible for the HSC/P mobilization phenotype, we evaluated the level of HSC/P mobilization after Ang-II administration in models of loss-of-function of Ang-II receptors. AT1R−/− and AT2R−/− mice were given a single dose of Ang-II and the level of circulating HSC/P was analyzed. While the deficiency of AT1R did not modify the effect of Ang-II on HSC/P mobilization, the single deficiency of AT2R completely prevented the increased HSC/P mobilization response after pharmacological administration of Ang-II (Fig. 2c), or in our model of chronic hydroangiotensinemia with Ang-II deficiency (Fig. 2d), indicating that AT2R is indispensable to mediate the mobilization of HSC/P induced by Ang-II.

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inhibition of all AT1R forms (AT1Ra and AT1R) on the progenitor mobilizing response to Ang-II at 1 min post-administration (Fig. 2e). The single deficiency of AT1R in the HM prevented Ang-II-mediated HSC/P mobilization (Fig. 2f), demonstrating that a non-cell-autonomous effect suffices to induce Ang-II/AT1R-dependent HSC/P mobilization. The deficiency of AT1R expression in the haematopoiesis of chimeric animals within a WT microenvironment, or the administration of the AT1R inhibitor, losartan, to chimeric WT mice did not prevent Ang-II-mediated mobilization of HSC/P, demonstrating that haematopoietic AT1R or pharmacological blockade of all forms of AT1R did not reverse the HSC/P mobilizing effect of Ang-II. However, the combination of a genetic deficiency of HSC/P AT1R and the specific pharmacological blockade of AT1R with losartan in chimeric mice prevented the HSC/P mobilizing effect of Ang-II (Fig. 2f), indicating the presence of a haematopoietic intrinsic effect on Ang-II-dependent HSC/P mobilization, which can only be prevented by a combined blockade of both AT1R and AT2R.

**AT2R activation induces HSC/P mobilization.** Pharmacological administration of the AT2R agonist C21 (ref. 30), which specifically binds to and activates AT2R but has different pharmacokinetic and pharmacodynamic temporal activity from Ang-II31, phenocopied the effect of Ang-II on the mobilization of haematopoietic progenitors (Fig. 2g and Supplementary Fig. 3d) and HSC (Fig. 2h and Supplementary Fig. 3e) by 4 h after administration. Administration of C21 did not modify endogenous levels of Ang-II (Supplementary Fig. 3f) and its effect was prevented by a deficiency of AT2R (Supplementary Fig. 3g), indicating that C21 acted through an on-target effect.

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**Figure 2 | Ang-II induces HSC/P de-adhesion and mobilization through AT2R signalling.** (a) Ang-II time kinetics of HSC/P mobilization. CFU-C of Ang-II-injected mice (blue) were compared with vehicle control-treated mice (open bars) at each time point. (b) PB CFU-C counts in mice submitted to continuous infusion of Ang-II for up to 7 days. (c) WT (black outlined bars), AT1R−/− (orange bars) and AT2R−/− (purple bars) primary mice were injected with PBS (−, solid bars) or Ang-II (+, hatched bars). n = 4 for each group. (d) CFU-C count in PB from WT, HyperAng-IIΔCx43-EC and AT2R-deficient-HyperAng-IIΔCx43-EC mice. (e) Schema of transplantation to generate AT2R-deficient H or HM mice. (f) Chimeric mice (H-WT, empty bars; H-WT AT2R−/−, hatched bars). The CFU-C counts were normalized to vehicle control (−, PBS). (g) Circulating CFU-C and CRU in PB from mice administered vehicle control (−, PBS, open bar) or C21 (4 mg kg−1, mosaic bar). (h) CFU-C counts. Values represent mean ± s.e.m. of three independent experiments with a minimum of 12 mice per group. (i) Adhesion of WT haematopoietic progenitors to EC from WT or HyperAng-IIΔCx43-EC mice. BM stromal cells from HyperAng-IIΔCx43-EC mice were used as a negative control. EC or stromal cells were treated with 100 μM Ang-II and the percentage of adhesion (blue bars) was compared with the adhesion of vehicle-treated cells (open bars). *P<0.05; **P<0.01. Student’s t-test for experiments that compare two groups or analysis of variance test with Bonferroni correction for experiments with more than two groups.
To identify whether Ang-II and the clinically used HSC/P mobilizer agent G-CSF collaborate in vivo, we treated C57Bl/6 mice with G-CSF (200 μg kg⁻¹ per day) for 5 days followed by a single injection of the AT₂R agonist C21, and determined their effect on the blood content of HSC/P using competitive repopulation and colony forming cell assays at 4 h after the administration of C21 on the 5th day of G-CSF administration. As shown in Supplementary Fig. 3h–i, no additive or negative effect was observed by the combination of G-CSF in either assay, which strongly suggested that G-CSF and Ang-II act through common signalling pathways. These data are in line with previous observations in mice where inhibition of Rac GTPase activity resulted in mobilization of HSC/P, but no additional effect of G-CSF on HSC/P mobilization was found.32,33

Nitric oxide (NO) is not responsible for HSC/P mobilization. HyperAng-II² CX43-EC mice have been shown to have increased plasma levels of NO (ref. 20) and Ang-II has been shown to induce NO release from EC34. NO levels also affect endothelial function35 and HSC release from endothelial niches36. To determine whether the regulation of NO levels in HyperAng-II² CX43-EC mice is implicated in HSC/P mobilization, we first analyzed and confirmed the presence of increased activation levels of nitric endothelial oxide synthase (eNOS) in ex-vivo propagated BM CD45⁻ Ter119⁻ CD31⁺ CD106⁻ BMEC from WT and HyperAng-II² CX43-EC mice (Supplementary Fig. 3). Then, we analyzed whether the exogenous administration of pharmacological doses of Ang-II or the NO scavenger, L-NG-nitroarginine methyl ester (L-NAME), to WT C57Bl/6 mice, resulted in changes in the levels of nitrates/nitrites and HSC/P in blood. Administration of either Ang-II or L-NAME reduced the plasma levels of nitrates/nitrites (Supplementary Fig. 3k) but L-NAME did not modify the circulating counts of HSC/P (Supplementary Fig. 3l). Similarly, the administration of the NO donor, 5-nitroso-N-acetylpenicillamine (SNAP), to WT C57Bl/6 mice did not result in changes in the count of PB HSC/P (Supplementary Fig. 3m). Altogether, this data indicates that plasma NO levels do not mediate Ang-II-dependent HSC/P mobilization.

Ang-II induces HSC/P de-adhesion from BMEC. Adhesion of HSC/P to the endothelial microenvironment is a basic mechanism of retention and homing of HSC/P within the BM that fits the response-time associated with the rapid effect of the administration of Ang-II. BMEC and BM stromal cells of mesenchymal origin have been reported to tightly control BM function3 and HSC release from endothelial niches37. To understand whether G-CSF and Ang-II perform an independent function35 and HSC release from endothelial niches36. To determine whether the regulation of NO levels in HyperAng-II² CX43-EC mice is implicated in HSC/P mobilization, we first analyzed and confirmed the presence of increased activation levels of nitric endothelial oxide synthase (eNOS) in ex-vivo propagated BM CD45⁻ Ter119⁻ CD31⁺ CD106⁻ BMEC from WT and HyperAng-II² CX43-EC mice (Supplementary Fig. 3). Then, we analyzed whether the exogenous administration of pharmacological doses of Ang-II or the NO scavenger, L-NG-nitroarginine methyl ester (L-NAME), to WT C57Bl/6 mice, resulted in changes in the levels of nitrates/nitrites and HSC/P in blood. Administration of either Ang-II or L-NAME reduced the plasma levels of nitrates/nitrites (Supplementary Fig. 3k) but L-NAME did not modify the circulating counts of HSC/P (Supplementary Fig. 3l). Similarly, the administration of the NO donor, 5-nitroso-N-acetylpenicillamine (SNAP), to WT C57Bl/6 mice did not result in changes in the count of PB HSC/P (Supplementary Fig. 3m). Altogether, this data indicates that plasma NO levels do not mediate Ang-II-dependent HSC/P mobilization.

Ang-II differently activates Rho GTPase in BMEC and HSC/P. In lung cancer-bearing mice, chronic cell-autonomous Ang-II/AT₁R signalling has been shown to amplify the splenic content of HSC/P and macrophage progenitors43. To analyze the cellular mechanism of rapid HSC/P de-adhesion from BMEC, we designed co-cultures of HSC/P with WT BMEC and analyzed the level of actin polymerization in HSC/P after the addition of Ang-II. As a source of HSC/P, BM c-kit⁺ /Sca-1 (LK) cells were used and allowed to adhere onto WT BMEC in the same manner as in Fig. 2i, and analyzed at different time points after Ang-II addition. Confocal microscopy of these co-cultures revealed reduced cortical localization of polymerized actin and an overall ~20% reduction in F-actin clustering in BM LK cells, as early as 5 min after Ang-II stimulation (Supplementary Fig. 4d,e), which closely preceded HSC/P de-adhesion in vitro and mobilization in vivo (as shown in Fig. 2a,i, respectively). Co-cultures of BM LK cells with BMEC demonstrated that the decrease in cortical actin polymerization of HSC/P required both AT₁R and AT₂R cell-autonomous signalling (Fig. 3f,g), suggesting that Ang-II signals through both AT₁R and AT₂R in HSC/P to disrupt the cytoskeletal integrity of adhered cells. Effector pull-down assays of Rho from isolated, adhered HSC/P, indicated that the activation of Rho in HSC/P was significantly decreased at 5 min after Ang-II stimulation (Fig. 3h), mirroring the Rho
GTPase activation signalling of BMEC on Ang-II stimulation. Cytoskeletal and Rho activity changes were associated with activation of Rac and inhibition of myosin light chain activation (Supplementary Fig. 4f,g). Phenocopying the loss of RhoA induced by Ang-II, genetic deletion of HSC/P RhoA activity in an inducible murine model (Supplementary Fig. 4h) resulted in HSC/P mobilization at levels similar to those of HyperAng-II^Apx43-EC mice, which was unresponsive to exogenous administration of Ang-II (Fig. 3i). This data strongly suggested that HSC/P Rho activity is required for Ang-II-dependent cell-autonomous HSC/P mobilization, and that the activation of HSC/P Rho signalling is dependent on both AT1R and AT2R.

Ang-II reduces β1-integrin activation on the cell membrane.

Impaired activation of β1-integrins is dependent on a fine regulation of Rho family GTPase activity and has been shown to be crucial for HSC/P adhesion to the BM microenvironment. Cytoskeletal and Rho/Rac activity changes induced by Ang-II were not associated with changes in the expression or localization of membrane BMEC or HSC/P β1-integrin (Supplementary Fig. 4i–l) but did impair membrane β1-integrin activation in BMEC (~65% inhibition, Fig. 3j,l) and more modestly in HSC/P (~10% inhibition, Fig. 3k,m). Overall, this data indicates that Ang-II induces cytoskeletal dysregulation in both BMEC and HSC/P in synchronous, mirror image processes of Rho family members. Similar to other cell types, the in vivo loss of Rac or...

**Figure 3** Ang-II alters Rho GTPase family activity and expression of activated β1-integrin. (a) Representative confocal microscopic image of F-actin (phalloidin, red) and nuclear (DAPI, blue) staining of 10 µM Ang-II-treated BMEC at different time points. BMEC were pretreated with 100 nM losartan or 1 µM PD123319 or vehicle control for 1 h before Ang-II exposure. Scale bar, 50 µm. (b) Measurement of morphological changes with cortical actin condensation in BMEC with exposure of vehicle control (open, no drug treated; orange, losartan; and purple, PD123319) or Ang-II (blue, no drug; hatched orange, losartan; and hatched purple, PD123319) at different time points as shown in Fig. 3a. (c) The diameter of all cells (cell width) was measured, and the average diameter of each field was calculated in Fig. 3b. (d,e) Representative immunoblot of effector binding domain pull-down assay for Rac (d) or Rho A (e) in BMEC with different lengths of Ang-II exposure. (f) Representative confocal microscopic image of F-actin (phalloidin, red) and nuclear (DAPI, blue) staining of LK after 5 min exposure of 100 nM Ang-II (+). Scale bar, 10 µm. (g) The mean fluorescence intensity (MFI) of phalloidin–rhodamine in LK treated with vehicle control (solid bars) or Ang-II (hatched bars) was measured in Fig. 3f. Losartan (100 nM, orange), PD123319 (1 µM, purple), combined losartan and PD123319 (orange/purple mosaic). (h) Representative immunoblot of effector binding domain pull-down assay for Rho A in LK cells with or without Ang-II treatment for 5 min. (i) CFU-C counts in PB from polyI:C-treated C57BL/6 WT (solid) and Rhoa^flox/flo (mosaic) mice treated with Ang-II (blue) or vehicle control (PBS, black outlined). Data represent mean ± s.e.m. A minimum of five mice were analyzed per group. (j,k) Representative FACS histograms of membrane-bound-activated membrane β1-integrin in BMEC (j) or LK (k) cells treated with vehicle control (red) or Ang-II (blue) and compared with isotype-treated cells (dotted line). (l,m) MFI of membrane-bound-activated membrane β1-integrin in BMEC (l) or LK (m) treated with vehicle control (open) or Ang-II (blue) was measured in Fig. 2j.k. A minimum of three independent experiments were performed. *P<0.05; **P<0.01; ***P<0.001; Student t-test for experiments that compare two groups or analysis of variance test with Bonferroni correction for experiments with more than two groups. For immunoblots, molecular weight markers in kilodalton are presented on the right side.
Rho activity decreased in decreased membrane-activated membrane β1-integrin expression, resulting in HSC/P–BMEC de-coupling and de-adhesion, suggesting that this is a highly conserved mechanism of cell adhesion.

**Augmented HSC/P mobilization in SCD is regulated by Ang-II.**

SCD results from substitution of a single nucleotide, valine for glutamic acid, at the sixth amino acid of the β-globin chain of hemoglobin A. SCD is characterized by globin polymerization that results in red cell dehydration, haemolysis and subsequent stress erythropoiesis. Vascular occlusions and pathology is a common feature in SCD patients. There is also an increased circulatory feature in SCD patients. There is also an increased circulatory ramifications of endogenous mobilization of HSC/P in SCD patients has not yet been delineated, however, it has been speculated that they may be a by-product of local inflammatory responses to the underlying vasculopathy prevalent in many organ sites in SCD patients. Based on the known pathobiological effects of SCD on endothelial function and our data regarding the role of Ang-II in HSC/P mobilization, we hypothesized that the increased circulation of HSC/P in SCD may be secondary to hyperangiotensinemia. To test this hypothesis, we analyzed PB specimens from SCD animals and patients. Berkeley sickle cell mice (Berk-SS) are used as animal models of human SCD, and exclusively express the human globins, human α- and β-globin, with deletions of the murine α- and β-globin genes. These mice recapitulate all the features found in humans with SCD, including irreversibly sickled red cells, anaemia and vasculopathy. Compared with WT mice (either Berk-AA or C57Bl/6 mice), Berk-SS mice had increased levels of Ang-II (Fig. 4a), which correlated with increased numbers of circulating HSC/P (Fig. 4b,c and Supplementary Figs 5a–d). A group of WT mice that had been transplanted with the BM of Berk-SS mice, also showed a significantly increased level of Ang-II compared with the WT cohorts, and when these Berk-SS/WT chimeras were administered the ACE inhibitor, captopril, the Ang-II concentrations were reduced to the level seen in WT controls (Fig. 4d). More interestingly, captopril-treated mice had an attenuated number of circulating HSC/P, compared with vehicle-treated Berk-SS/WT chimeric mice (Fig. 4e). Mechanistically, similar to our experiments in pharmacological or genetic models of hyperangiotensinemia, we analyzed whether the deficiency of microenvironment AT2R had any effect on the mobilization of HSC/P in Berk-SS chimeric mice. Berk-SS BM was transplanted into fully myeloablated WT or AT2R+/− mice to generate chimeric animals (Supplementary Fig. 5e). All chimeric Berk-SS mice were anaemic and showed all the haematological characteristics of primary Berk-SS mice by 6 weeks post-transplantation. Exogenous administration of Ang-II maintained its HSC/P mobilizing effect in Berk-SS/WT chimeric mice, but not in chimeric Berk-SS/AT2R+/− mice (Supplementary Fig 5f), confirming the requirement of AT2R expression for Ang-II-dependent mobilization in SCD mice.

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Figure 4 | Increased Ang-II level correlates with increased HSC/P circulation in SCD. (a) Plasma Ang-II concentration in Berkeley AA (WT, black) and Berk-SS (red) mice. **P < 0.001. Data represent mean ± s.e.m. (b) CFU-C in PB from Berkeley AA (WT, black) and Berk-SS (red) mice. (c) Long-term HSC (LT-HSC) in PB from WT and Berk-SS mice. (d) Plasma Ang-II concentration in C57Bl/6 mice transplanted with either the BM of AA (WT, black, n = 8) or SS mice (red, n = 7). Subgroup of chimeric SS mice were treated with captopril (blue, n = 7). (e) CFU-C counts in PB from the mice in c. Data represent mean ± s.e.m. (f) Ang-II concentration in plasma from age-matched, unaffected sibling control (black, n = 8), patient with SCD (red, n = 15) and SCD patient with lisinopril treatment (blue, n = 4). *P < 0.05. Data represent mean ± s.e.m. (g) Number of circulating CD34+ cells in the PB from f. Data represent mean ± s.e.m. (h) CFU-C counts in PB from g a healthy age-matched, unaffected sibling controls (black), patients with SCD (red) and SCD patients in treatment with lisinopril (blue). Values represent mean ± s.e.m. of three independent experiments. *P < 0.05. **P < 0.01, ***P < 0.001. Student t-test for experiments that compare two groups, or analysis of variance test with Bonferroni correction for experiments with more than two groups.
To ensure that the phenomenon seen in sickle mice also occurs in humans with SCD, we analyzed the levels of Ang-II and the counts of circulating HSC/P in SCD patients and then compared them to unaffected sibling controls with both normal β-globin alleles. All plasma Ang-II levels (Fig. 4I), circulating numbers of CD34+ cells (Fig. 4G) and CFU-C in PB (Fig. 4H) were increased in SCD patients, and treatment with another ACE inhibitor, lisinopril, resulted in significantly decreased plasma levels of Ang-II and was associated with a corresponding 50–70% reduction in the circulating blood progenitors (Fig. 4f–h).

Discussion
Following a step-by-step approach and combining genetic and pharmacological methods, our manuscript provides a set of compelling data, demonstrating the role of high levels of plasma Ang-II in HSC/P mobilization. Mice with chronic hyperangiotensinemia secondary to vasculopathy have a two to three fold increase in the number of circulating myeloid committed haematopoietic progenitors and repopulating stem cells. The increase in circulating HSC/P accounts for ~0.1% CRU and ~0.5% CFU-C, respectively, of all BM HSC/P. These levels are similar to the mobilization with the CXCR4 inhibitor, AMD3100 (ref. 15). Using combinations of AT receptor knockout mice and pharmacological inhibitors, our data support a distinct role for AT1R in BMEC actin polymerization, which would coordinate with decreased cortical polymerized actin in HSC/P through either AT1R or AT2R. Ang-II would trigger distinct signalling pathways in HSC/P and BMEC that result in a similar outcome, which is the downregulation of activated membrane β1-integrin and HSC/P de-adhesion from their attachment to BMEC. The effect of Ang-II on microenvironmental cells seems to be quite specific, since Ang-II does not modify HSC/P adhesion to other cell types such as BM mesenchymal lineage cells.

The translational implications of this work are self-evident. Every year, millions of patients receive anti-angiotensin therapies due to the harmful effects associated with chronic hyperangiotensinemia in cardiac, renal, or liver failure. While the ultimate fate of circulating HSC/P remains incompletely understood, there is an abundance of information on their role in innate immune regulation and tissue regeneration. Circulating HSC/P can survey peripheral organs and foster the local production of tissue-resident innate immune cells under steady-state conditions and in response to inflammatory signals9,34. An example of haematological vasculopathy is SCD, where vascular occlusions and pathology are a common feature10. In these patients, an increased circulatory count of primitive HSC/P is well-documented11 in the context of sustained leukocytosis, which remains a major cause of inflammation associated morbidity10,22–34. The absence of a relationship between NO levels and Ang-II-dependent HSC/P mobilization suggests that hyperangiotensinemia together with NO acting on HSC release from endothelial niches36, may act to mobilize HSC/P via different pathways that are dependent on cell context and induce divergent signalling pathways.

The effects of Ang-II on cytoskeletal dysregulation of HSC/P and BMEC seem to be independently causative of HSC/P mobilization. Other possible effects of hyperangiotensinemia were found not responsible for the mobilizer effect of Ang-II. Increased vascular tone and modulation of NO levels are known effects of hyperangiotensinemia27,34. However, our data rule out the vasopressor or NO mediator effect as the mechanisms responsible for Ang-II-dependent HSC/P mobilization. β-adrenergic stimulation has also been shown to be critical at controlling HSC/P egress24. We did not observe any significant changes in the levels of norepinephrine or epinephrine in the BM or blood of mice with chronic hyperangiotensinemia, nor did the β-adrenergic blocker propranolol have an effect on their circulating HSC/P counts, suggesting that in the context of vasculopathy, sympathetic nervous system blockade may not counteract the effect of Ang-II.

Our data indicate that hyperangiotensinemia induces HSC/P de-adhesion and mobilization through a direct effect on AT receptors in a cell-autonomous and non-cell-autonomous manner. HSC/P mobilization depends on the expression of AT1R in the endothelium, but not in the mesenchymal stroma of the BM microenvironment. Ang-II signals through BMEC AT1R, and through both AT1R and AT2R in HSC/P, resulting in distinct changes in RhoA activation and in cytoskeletal rearrangements of both BMEC and HSC/P, which results in common down-regulation of the levels of active membrane β1-integrin, and HSC/P de-adhesion and mobilization (Fig. 5). Gene deletion of RhoA phenocopies the mobilization effect of Ang-II, and RhoA-deficient mice become insensitive to Ang-II, suggesting that RhoA activation is indispensable for Ang-II-dependent mobilization.

Altogether, this data indicates that hyperangiotensinemia/AT1R signalling plays a homoeostatic role in the control of the number of circulating progenitors in vasculopathies in general, and in SCD in particular. These studies demonstrate that acute or chronic hyperangiotensinemia results in HSC/P mobilization and represents a common pathway controlling HSC/P traffic in disease. Targeting Ang-II generation in vasculopathies and SCD may have a detrimental effect on the regenerative roles of circulating stem cells and progenitors, which, clinicians should take into account when using anti-angiotensin therapies.

Methods
Animals. All animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all protocols were approved by institutional care and use committees for animal research at the
Cincinnati Children's Hospital Research Foundation. Endothelial specific-Cx43-deficient mice were generated by crossing Tek promoter-Cre recombinase transgenic mice with Cx43 flox/flox mice56. Cx43 flox/flox mice56 were used, in which inactivation of RhoA in the whole BM as well as haematopoietic cells was induced by intraperitoneal administration of double-stranded RNA polyI:C (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at a dose of 10 mg kg\(^{-1}\) i.p., two doses every other day. These mice had been backcrossed for a minimum of five generations. AT\(_{1}\)Ra (AT\(_{1}\)R) knockout mice57 were obtained from Jackson Laboratories, Bar Harbor, ME, USA. AT\(_{1}\)R knockout mice57 were kindly provided by Dr. Inagami (Vanderbilt University School of Medicine, Nashville, TN, USA). TekCre/Cx43floxflox/AT\(_{1}\)R knockout mice57 and their genetic littermate controls were generated by crossing TekCre/Cx43floxflox/ mice with AT\(_{1}\)R knockout mice. Ubiquitin C-EGFP mice have been generated previously58. These mice had been backcrossed for > 40 generations into C57Bl/6 mice. Berk-Ss mice48 were backcrossed for four generations into C57Bl/6 background to allow survival through adulthood. Experimental transgenic animals were male or female with ages ranging from 6 to 20 weeks. Six to eight-week-old female WT C57Bl/6 mice were obtained commercially (Jackson Laboratory; Harlan Laboratories, Frederick, MD, USA) and used as WT donors and/or recipients of transduction-transplantation models.

**Human specimens.** PB specimens from healthy normal volunteers were obtained through Institutional Review Board-approved protocols and donor informed consents from Cincinnati Children's Hospital Medical Center and the University of Cincinnati. In pregnant women, the specimens were uninfected with unanticipated SCD in different phase (age ≥ 6 years of age) were collected through Institutional Review Board-approved protocols and donor informed consent from Cincinnati Children's Hospital Medical Center and the University of Cincinnati.

**Flow cytometry analysis and sorting.** For the phenotypical characterization of HSC/P, low density (LD) BM cells were obtained after separation using Histopaque-10777 (Sigma-Aldrich, St. Louis, MO, USA) and Ficoll-Paque gradient (GE Healthcare). Lin\(^{-}\) cells were purified using CD45 and Ter119 antibodies to identify the Lin\(^{-}\) population. IL7R (PE Cy7 conjugated, clone SB/199), CD34 (Pacific blue, clone Sph-4), CD45R (B220; clone RA3-6B2), Gr-1 (Ly-6G and Ly-6C; clone RB6-8C5), CD4 (L3T4; clone RM4-5), CD8a (Ly-2; clone 53-6.7), CD3e (clone 145-2C11), CD11b (M1/70) and Ter119 (Ly-76), allophycocyanin (APC)-conjugated c-Kit and fluorescein isothiocyanate (FITC)- conjugated Sca-1 antibodies to identify the Lin\(^{-}\) c-Kit\(^{+}\)/Sca-1\(^{+}\) (LSK) BM cell population. IL7R (PE Cy7 conjugated, clone SB/199), CD34 (Pacific blue, clone RAM34), CD16/CD32 (PerCP-Cy5.5 conjugated, clone 2.4G2) and CD135 (PE conjugated, clone A2F10) were used. A sequential gating strategy on nucleated cells as discriminated by light scatter properties and sequential gating of lineage negative LSK were defined as LSK/CD34\(^{+}\)/CD135\(^{-}\) – short-term HSC were defined as LSK/CD34\(^{+}\)/CD135\(^{-}\) – multipotential progenitors were defined as LSK/CD34\(^{+}\)/CD135\(^{-}\).

For more differentiated progenitors, megakaryoblastic-erythroid progenitors were defined as LK/CD34\(^{-}\)/CD16/CD32\(^{-}\) – common myeloid progenitors were defined as LK/CD34\(^{-}\)/CD16/CD32\(^{-}\) – granulocytemacrophage progenitors were defined as LK/CD34\(^{-}\)/CD16/CD32\(^{-}\) – and granulocyte-macrophage progenitors were defined as LK/CD34\(^{-}\)/CD16/CD32\(^{-}\) – events. For sorting of BM-derived ECs, PE conjugated CD45 and Ter119 antibodies were used along with APC-conjugated CD31 (clone MEC 13.3) and FITC-conjugated CD106 (clone 429) and sorted by flow cytometry (FACSaria II, BD Biosciences, San Jose, CA, USA). Rhodamine-phalloidin (Invitrogen), PE conjugated 

**Immunofluorescence and confocal microscopy imaging.** For confocal imaging, the expected electrophoretic mobility for each protein molecular weight. Complete, uncropped images of Western blots used for figure illustrations are provided in the Supplementary Fig. 6.

**Western blotting and pull-down assays.** Lineage negative cells were purified using magnetic bead-based methods (Miltenyi Biotec, Bergisch Gladbach, Germany), and protein extracts were prepared using a radioimmunoprecipitation lysis buffer (RIPA, Cell Signaling Technology, Danvers, MA, USA), or for pull-down assays a high-Mg\(^{2+}\) containing buffer supplemented with protease and phosphatase inhibitors cocktails following manufacturer’s instructions (Roche Applied Science, Mannheim, Germany). GTPase activities as well as protein expression and phosphorylation were measured as previously described by our group59 by effect binding precipitation with p21-activated kinase-bound beads (for Rac activity) and rho-associated bound beads (for Rho activity). The lysates were separated using 10% SDS-PAGE gels, transferred to polyvinylidyne fluoride membranes and detected with antibodies. The primary antibodies used were: β-actin (1:6,000, clone AC-15, Sigma-Aldrich), AT\(_{1}\)R (1:3,000, clone 1E10-1A9, Abcam, Cambridge, MA), AT\(_{2}\)R (1:5,000, clone EP10878, Abcam). Antibodies against eNOS (polyclonal, 1:1,000), phospho-eNOS (1:1,000, clone C93C), MLC (polyclonal, 1:1,000), p-MLC (Ser19, polyclonal, 1:3,000), collagen (1:1,000, clone D59) and p-cofilin (1:1,000, clone 77G2; Ser3) were obtained from Cell Signalling, and Cx43 (1:500, clone CX-B1, Invitrogen), Rac (1:2,000, Rho (1:1,000) and VEGFR2 (1:500) were obtained from Millipore Corp., Billerica, MA. Gels were visualized using autoradiography and band density was analyzed (Image J software, National Institutes of Health, USA) at the expected electrophoretic mobility for each protein molecular weight. Complete, uncropped images of Western blots used for figure illustrations are provided in the Supplementary Fig. 6.

**In vivo drug administration.** All inhibitors and antagonists were given by intraperitoneal injection. Ang II (Sigma-Aldrich, 1.44 mg kg\(^{-1}\)) and enalapril (Sigma-Aldrich, 1 mg kg\(^{-1}\)) were injected and PB was drawn at 10 min post-administration61. Acetic oxygenated buffer dissolved SNAP (Cayman Chemical Co., Ann Arbor, MI, USA) was diluted in PBS (10 mg kg\(^{-1}\)) and was collected after 10 min post-administration. Propranolol was dissolved in water (Sigma-Aldrich, 10 mg kg\(^{-1}\)) and then administered in mice intraperitoneally. For each Western blotting and pull-down assays. Lineage negative cells were purified using magnetic bead-based methods (Miltenyi Biotec, Bergisch Gladbach, Germany), and protein extracts were prepared using a radioimmunoprecipitation lysis buffer (RIPA, Cell Signaling Technology, Danvers, MA, USA), or for pull-down assays a high-Mg\(^{2+}\) containing buffer supplemented with protease and phosphatase inhibitors cocktails following manufacturer’s instructions (Roche Applied Science, Mannheim, Germany). GTPase activities as well as protein expression and phosphorylation were measured as previously described by our group59 by effect binding precipitation with p21-activated kinase-bound beads (for Rac activity) and rho-associated bound beads (for Rho activity). The lysates were separated using 10% SDS-PAGE gels, transferred to polyvinylidyne fluoride membranes and detected with antibodies. The primary antibodies used were: β-actin (1:6,000, clone AC-15, Sigma-Aldrich), AT\(_{1}\)R (1:3,000, clone 1E10-1A9, Abcam, Cambridge, MA), AT\(_{2}\)R (1:5,000, clone EP10878, Abcam). Antibodies against eNOS (polyclonal, 1:1,000), phospho-eNOS (1:1,000, clone C93C), MLC (polyclonal, 1:1,000), p-MLC (Ser19, polyclonal, 1:3,000), collagen (1:1,000, clone D59) and p-cofilin (1:1,000, clone 77G2; Ser3) were obtained from Cell Signalling, and Cx43 (1:500, clone CX-B1, Invitrogen), Rac (1:2,000, Rho (1:1,000) and VEGFR2 (1:500) were obtained from Millipore Corp., Billerica, MA. Gels were visualized using autoradiography and band density was analyzed (Image J software, National Institutes of Health, USA) at the expected electrophoretic mobility for each protein molecular weight. Complete, uncropped images of Western blots used for figure illustrations are provided in the Supplementary Fig. 6.

**Immunofluorescence and confocal microscopy imaging.** The confocal images of the BMECs treated with 10 µM angiotensin for different time periods were acquired using LSM 710 (Carl Zeiss, Thornwood, NY) confocal system at ×10 optical magnification. The merged images of rhodamine-phalloidin and DAPI are presented. At 0, 5, 15 minutes Ang-II treatment, of angiotensin treatment, the cells that have morphological changes with condensation of actin filaments in the cortical region are counted, and presented as percentage of total cells in the field. At least 10 fields for each time point were counted. The diameter (width) of the cells at each of the time points was measured using ImageJ software, National Institutes of Health, USA at the expected electrophoretic mobility for each protein molecular weight. Complete, uncropped images of Western blots used for figure illustrations are provided in the Supplementary Fig. 6.
time point, 8–10 fields of images were measured, and therefore a total of 600–800 cells were measured for the quantification of the diameter of the cells at each time point of angiotensin treatment. Bar, 50µm. Sorted LK cells stained with carboxyfluorescein succinimidyl ester (1 M, Invitrogen) or from ubiquitin C-EGFP mice were co-cultured with WT BMEC for 4 h in absence of other additives and for another an additional hour in absence or presence of 100 nM losartan, 1 M PD123319 (Tocris Bioscience, Bristol, UK) or both. At the end of the incubation period, gel II was added for 5 min, before any significant His/C-P detachment occurs, and cultures were fixed with 4% formaldehyde. The stained cells were imaged on a confocal microscope (Observer Z1, Carl Zeiss) using a Plan Apochromat objective, and were processed using Adobe Photoshop v7 (Adobe Systems Inc., San Jose, CA).

**Statistical analysis.** Quantitative data is given as mean ± s.e.m. Statistical significance was determined using an unpaired Student t-test or one-way analysis of variance with Bonferroni correction. A value of P<0.05 was considered to be statistically significant.

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**Author contributions**

K.H.C., Y.Z., P.M. and J.A.C. designed the experiments. K.H.C., Y.Z., P.M. and J.A.C. wrote the manuscript. A.M.W., K.Y.B., M.P., S.E.H., A.L. and X.Z. performed the experiments. P.M., Y.Z., K.H.C., R.C.N, S.R., A.P., A.M.W., K.Y.B., M.P., S.E.H., A.L. and X.Z. analyzed the data. K.H.C., Y.Z., P.M. and J.A.C. wrote the manuscript.

**Additional information**

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

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