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Cardiac angiogenic imbalance leads to peripartum cardiomyopathy

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Peripartum cardiomyopathy (PPCM) is an often fatal disease that affects pregnant women who are near delivery, and it occurs more frequently in women with pre-eclampsia and/or multiple gestation. The aetiology of PPCM, and why it is associated with pre-eclampsia, remain unknown. Here we show that PPCM is associated with a systemic angiogenic imbalance, accentuated by pre-eclampsia. Mice that lack cardiac PGC-1α, a powerful regulator of angiogenesis, develop profound PPCM. Importantly, the PPCM is entirely rescued by pro-angiogenic therapies. In humans, the placenta in late gestation secretes VEGF inhibitors like soluble FLT1 (sFLT1), and this is accentuated by multiple gestation and pre-eclampsia. This anti-angiogenic environment is accompanied by subclinical cardiac dysfunction, the extent of which correlates with circulating levels of sFLT1. Exogenous sFLT1 alone caused diastolic dysfunction in wild-type mice, and profound systolic dysfunction in mice lacking cardiac PGC-1α. Finally, plasma samples from women with PPCM contained abnormally high levels of sFLT1. These data indicate that PPCM is mainly a vascular disease, caused by excess anti-angiogenic signalling in the peripartum period. The data also explain how late pregnancy poses a threat to cardiac homeostasis, and why pre-eclampsia and multiple gestation are important risk factors for the development of PPCM.

PPCM affects 1 in 300 to 1 in 3,000 pregnancies, with geographic hot spots of high incidence, such as Nigeria and Haiti[1,2]. The disease is characterized by systolic heart failure presenting in the last month of pregnancy or the first 5 months post-partum. Although approximately half of affected women recover cardiac function post-partum, many patients progress to chronic heart failure, cardiac transplantation or death. Thus, PPCM can devastate otherwise healthy young women and their infants. PPCM remains a disease of unknown aetiology. The onset late in gestation does not coincide with increased haemodynamic load on the heart, suggesting that other mechanisms are responsible. Recent data have suggested that anti-angiogenic prolactin fragments may have an important role in causing the disease in some patients[3]. Risk factors for PPCM also include pre-eclampsia and multiple gestation, suggesting potential mechanistic overlap with these processes[1,2].

PGC-1α is a transcriptional coactivator that drives mitochondrial biogenesis and other metabolic programs in many tissues, including the heart[4,5]. PGC-1α is highly expressed in the heart, and mice lacking PGC-1α globally have abnormal cardiac energetic reserves and respond poorly to stressful stimuli such as transverse aortic banding[6,7]. In addition to its role in mitochondrial homeostasis, PGC-1α also induces the expression and secretion of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), which leads to formation of new blood vessels[8,9]. Although the angiogenic function of PGC-1α has been described in skeletal muscle, its role in cardiac tissue remains unexplored.

Cardiac-specific PGC-1α deletion leads to PPCM

To study further the role of PGC-1α in the heart, we generated cardiac-specific PGC-1α knockout (HKO) mice (see Methods). While studying these mice, we noticed that female HKO mice were fertile, and delivered normal litter sizes (not shown), but invariably died after one or two pregnancies (Fig. 1a). The hearts of these mice were large, dilated and fibrotic (Fig. 1b–d), consistent with a dilated cardiomyopathy. Two-dimensional M-mode echocardiography revealed dilated, poorly contractile hearts in HKO mice after their second delivery (Fig. 1e). Left ventricular end-diastolic dimensions (LVEDD) and left ventricular end-systolic dimensions (LVESD) were markedly enlarged, and fractional shortening, a direct measure of cardiac contractile function, was profoundly depressed (Fig. 1f–i). Nulliparous mice, as well as post-partum control mice, were not affected. Males were also not affected (Supplementary Fig. 1). Thus, the absence of PGC-1α in cardiomyocytes leads to a profound PPCM in mice.

PGC-1α regulates angiogenesis in cardiac tissue

We have recently shown in skeletal muscle that PGC-1α regulates angiogenesis by driving the expression of angiogenic factors like VEGF[8,9]. Anti-angiogenic therapies, including antibodies that neutralize VEGF and small-molecule VEGF receptor inhibitors, are increasingly being used in oncological and ophthalmological treatments, and cardiomyopathy and heart failure have recently been recognized as important side effects[10,11], showing that anti-angiogenic therapy can be harmful to the heart in humans. Impaired VEGF signalling has also been linked with cardiac dysfunction in mice[12,13]. At the same time, late pregnancy is a strong anti-angiogenic environment, partly owing to the secretion by the placenta of anti-angiogenic factors, like sFLT1, that bind to and neutralize soluble members of the VEGF family[14]. These observations led us to postulate that PGC-1α regulates an angiogenic

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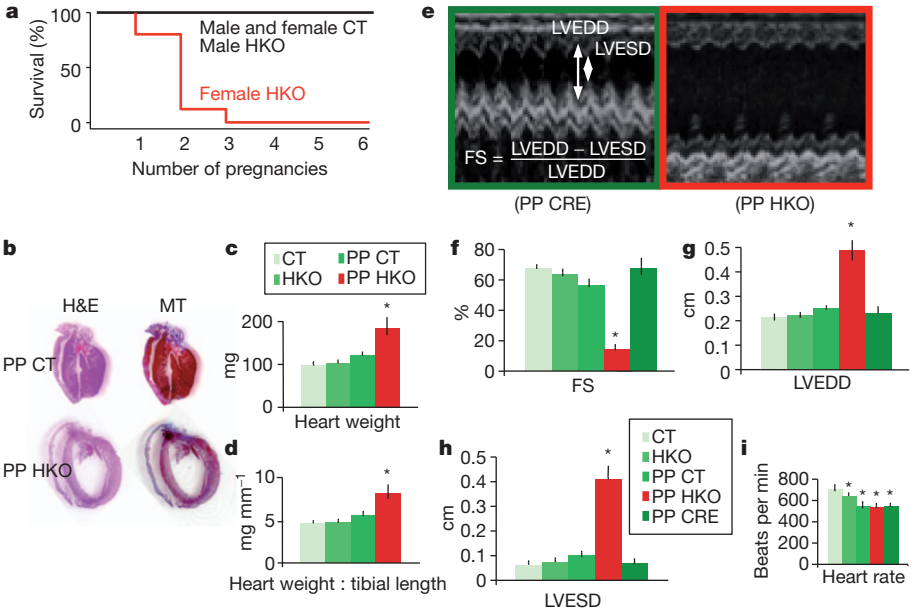


Figure 1 | Mice lacking cardiac PGC-1α develop peri-partum cardiomyopathy. **a**, Kaplan–Meier survival curve in female αMHC-Cre: PGC-1α$^{lox/lox}$ mice (HKO) versus male HKO mice or control mice (CT) of either gender. **b**, Haematoxylin and eosin and Masson's trichrome stains of hearts from post-partum HKO mice (PP HKO), versus post-partum CT mice (PP CT). **c**, **d**, Heart weight (**c**) and heart weight:tibial length ratios (**d**) of nulliparous CT and HKO mice, and PP CT and HKO mice. **e**, Sample M-mode echocardiograms of PP HKO mice and control mice containing the αMHC–Cre transgene alone (PP CRE). **f–i**, Echocardiographic measures in mice of the indicated genotypes, either nulliparous or post-partum. $n \geq 5$ for all groups. $^*P < 0.05$.

program in cardiomyocytes, and that the absence of this program would leave the hearts defenceless to the anti-angiogenic setting of late pregnancy, thus leading the animals to develop PPCM.

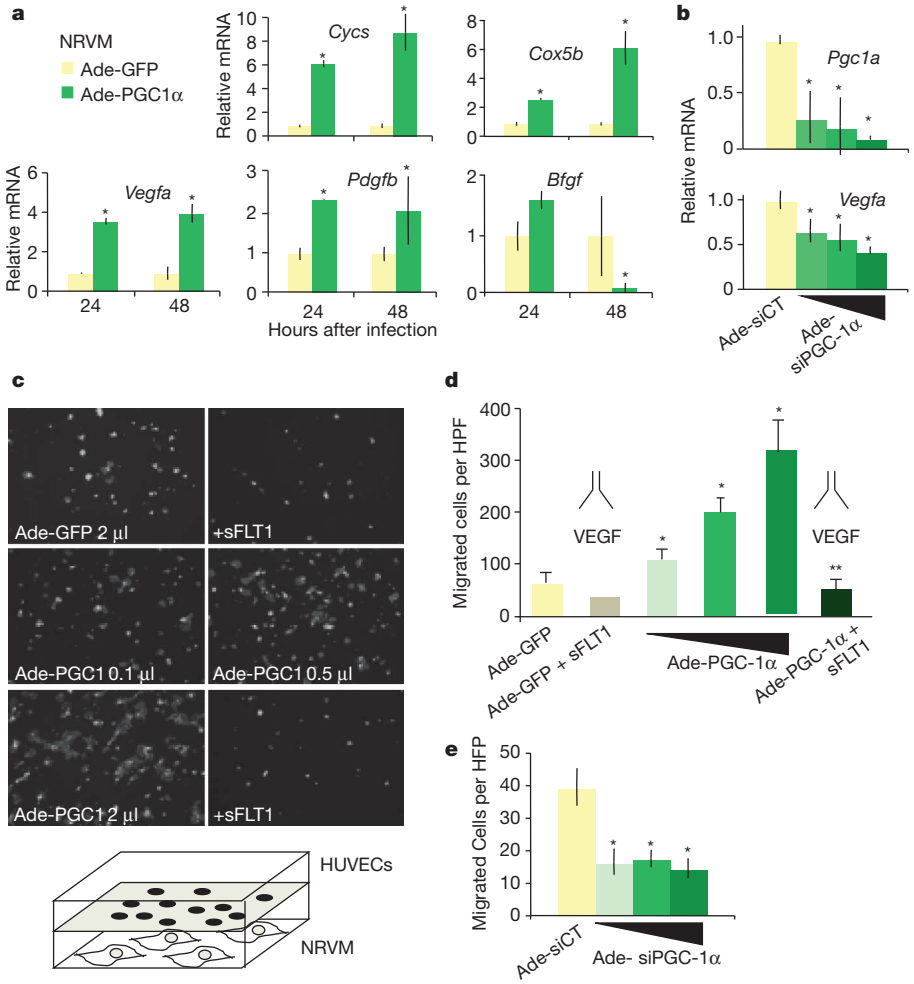
Consistent with this idea, overexpression of PGC-1α in neonatal rat ventricular myocytes (NRVMs) strongly induced angiogenic genes involved in the activation and recruitment of endothelial cells (for example, *Vegfa*) and mural cells (for example, *Pdgfb*), as well as genes that are involved in the mitochondrial respiratory chain[15] (for example, *Cycs* and *Cox5b*) (Fig. 2a). Interestingly, some angiogenic genes were repressed (for example, *Bfgf*), with a pattern of repression that is similar to that seen in skeletal muscle cells[8], indicating that PGC-1α reprograms the angiogenic program in a stereotypical manner. Conversely, PGC-1α short interfering RNA (siPGC-1α) significantly suppressed the expression of *Vegfa* (Fig. 2b). Endothelial activation and migration is a hallmark of angiogenesis. As shown in Fig. 2c, d, overexpression of PGC-1α in NRVMs led to a marked, dose-dependent, up to sixfold increase in the migration of the adjacent human umbilical vein endothelial cells (HUVECs) in a co-culture system. Addition of sFLT1 completely neutralized the induced endothelial migration, indicating that secreted members of the VEGF family, probably VEGFA itself, are critical for the effect. Conversely, repression of PGC-1α in NRVMs by siRNA significantly repressed endothelial migration (Fig. 2e). Thus, PGC-1α controls an angiogenic program in cardiomyocytes.

To test whether PGC-1α is required for this program in intact animals, levels of *Vegfa* and other angiogenic factors were measured in hearts from PGC-1α HKO mice. The expression of *Vegfa* and *Pdgfb*, and a number of other angiogenic factors, was repressed in these hearts by as much as 50% (Fig. 3a). VEGFA protein was decreased by 30% in HKO hearts (Fig. 3b). Levels of VEGFA are normally tightly regulated, and even global haplo-insufficiency is lethal[16,17], underscoring the significance of a 30% drop in protein levels. Consistent with these findings, the capillary density of HKO hearts, as measured by staining with the endothelial-specific marker CD31, was decreased by about 15% (Fig. 3c, d). Thus, PGC-1α regulates both vascular density (these data) and mitochondrial function[18] in the heart, providing an important regulatory link between the delivery of fuel (through blood vessels) and its consumption (by mitochondria).These data suggest that PPCM in these mice might be caused by the combination of a heart-specific vascular defect caused by the absence of PGC-1α, and the normal systemic anti-angiogenic environment of late pregnancy. Indeed, the vascular density in HKO hearts decreased by almost 50% post-partum (Fig. 3c, d), which is equivalent to the decrease in vascular density seen in mice lacking cardiac VEGF[19]. Consistent with this vascular rarefaction, perfusion of post-partum HKO hearts was profoundly decreased by nearly 50% compared to wild-type animals, as determined by methoxyisobutylisonitrile (MIBI) uptake and single-photon emission computed tomography and X-ray computed tomography (SPECT/CT) (Fig. 3e, f).

Pro-angiogenic therapy rescues PPCM

To test directly the idea that an angiogenic imbalance drives PPCM in HKO mice, rescue experiments were carried out. In a first series of experiments, breeding mice were administered daily subcutaneous injections of VEGF121 protein (100 μg kg^{-1}), an isoform of VEGFA, versus vehicle control (Supplementary Fig. 2). The efficacy of VEGF121 injections was confirmed by the presence of VEGF121 in the serum, and robust activation of cardiac VEGFR2 phosphorylation within 30 min of injection (Supplementary Fig. 2b, c). The VEGF treatment led to improved survival of the breeding HKO females; they were able to survive up to five pregnancies (Supplementary Fig. 2d). However, capillary density was only partly rescued, cardiac contractility was only marginally improved and the hearts remained enlarged (Supplementary Fig. 2e–g). Thus, VEGF administration partly rescued lethality in multiparous HKO animals, but it was insufficient to fully rescue PPCM, suggesting that other pathways are also important.

It has recently been shown that STAT3 regulates mitochondrial superoxide dismutase (MNSOD) and protects against reactive oxygen species (ROS) in the heart[3]. Absence of cardiac STAT3 and the consequent increase in ROS led to inappropriate cleavage of prolactin to a potent anti-angiogenic 16-kDa form, and subsequent PPCM (Fig. 4a). Importantly, the PPCM could be rescued by treatment with bromocriptine, which inhibits the secretion of prolactin



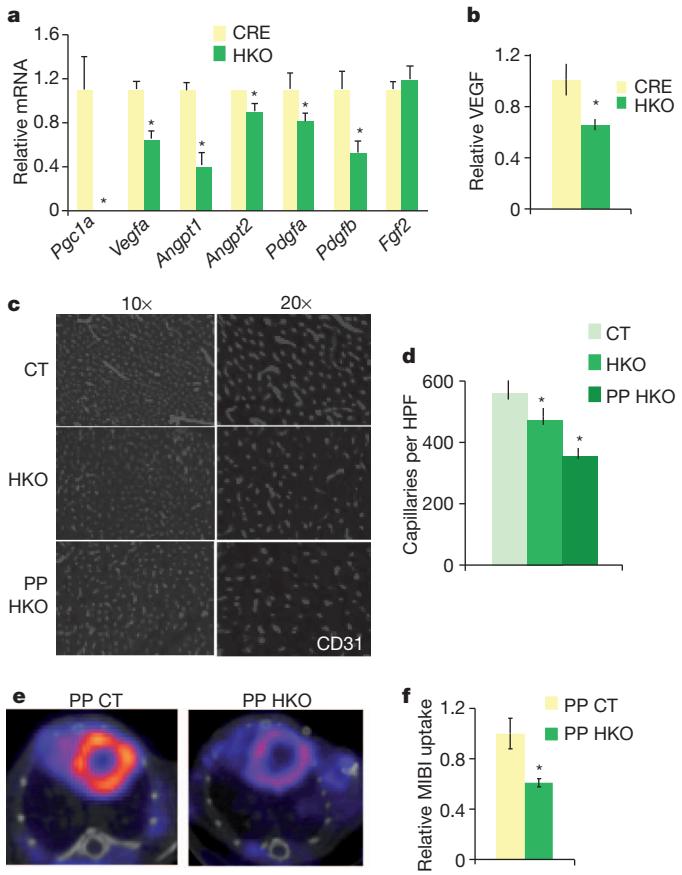


Figure 2 | PGC-1α regulates an angiogenic program in cardiomyocytes. **a, b**, Relative expression of mitochondrial genes (*Cycs* and *Cox5b*) and angiogenic genes (*Vegfa*, *Pdgfb* and *Bfgf*) in NRVMs infected with adenovirus expressing PGC-1α (Ade-PGC-1α) versus adenovirus expressing green fluorescent protein (Ade-GFP) (**a**) or siPGC-1α versus siCT (**b**). **c,** PGC-1α expression in NRVMs induces the migration of adjacent endothelial cells, and the migration is blocked by sFLT1. Representative images show phalloidin-stained endothelial cells that have migrated towards the NRVMs. The experimental procedure using the Transwell migration chamber is shown in the bottom panel. **d**, Data from the procedure in **c** are quantified. HPF, high power field. **e**, Knockdown of PGC-1α inhibits the migration of endothelial cells. Error bars are ± s.e.m. $*P < 0.05$ versus control. $**P < 0.05$ versus cells not treated with sFLT1.

from the pituitary gland. PGC-1α is known to increase ROS scavenging[20]. In cardiomyocytes, overexpression of PGC-1α strongly induced *Mnsod* mRNA and protein expression (Supplementary Fig. 3a, b). Conversely, MNSOD was repressed in PGC-1α HKO hearts, and levels of ROS were increased (Supplementary Fig. 3c, d). Thus, PGC-1α and STAT3 both regulate MNSOD and ROS in cardiomyocytes, suggesting that the absence of PGC-1α in the heart may, like the absence of STAT3, lead to prolactin-mediated anti-angiogenic effects. Prolactin had no direct effects on PGC-1α or VEGF expression in cardiac cells, and prolactin levels did not differ in heart or serum between wild-type and HKO animals (Supplementary Fig. 4).

These observations suggest that PGC-1α regulates two separate pro-angiogenic pathways in the heart—a VEGF pathway and a prolactin pathway—and that aberration of both pathways in PGC-1α HKO mice leads to PPCM (Fig. 4a). To test this idea directly, both pathways were rescued simultaneously: breeding HKO mice were treated with daily subcutaneous injections of VEGF protein and with bromocriptine supplementation in the water (Fig. 4b). This double treatment resulted in complete rescue of the PPCM in HKO females

Figure 3 | Mice lacking cardiac PGC-1α have reduced microvascular density that is worsened by pregnancy. **a, b**, Relative mRNA expression of several angiogenic factors (**a**) and VEGF protein levels (**b**) in HKO versus CRE control hearts. **c, d**, Vascular density in hearts from nulliparous or post-partum CT and HKO mice. Representative images stained for CD31 (also known as PECAM) are shown (**c**) and quantified (**d**). **e, f**, Reduced cardiac MIBI uptake in PP HKO versus PP CT animals. Representative SPECT/CT images are shown (**e**), and quantified (**f**). $n \geq 5$ for all groups. Error bars are ± s.e.m. $*P < 0.05$ versus control.

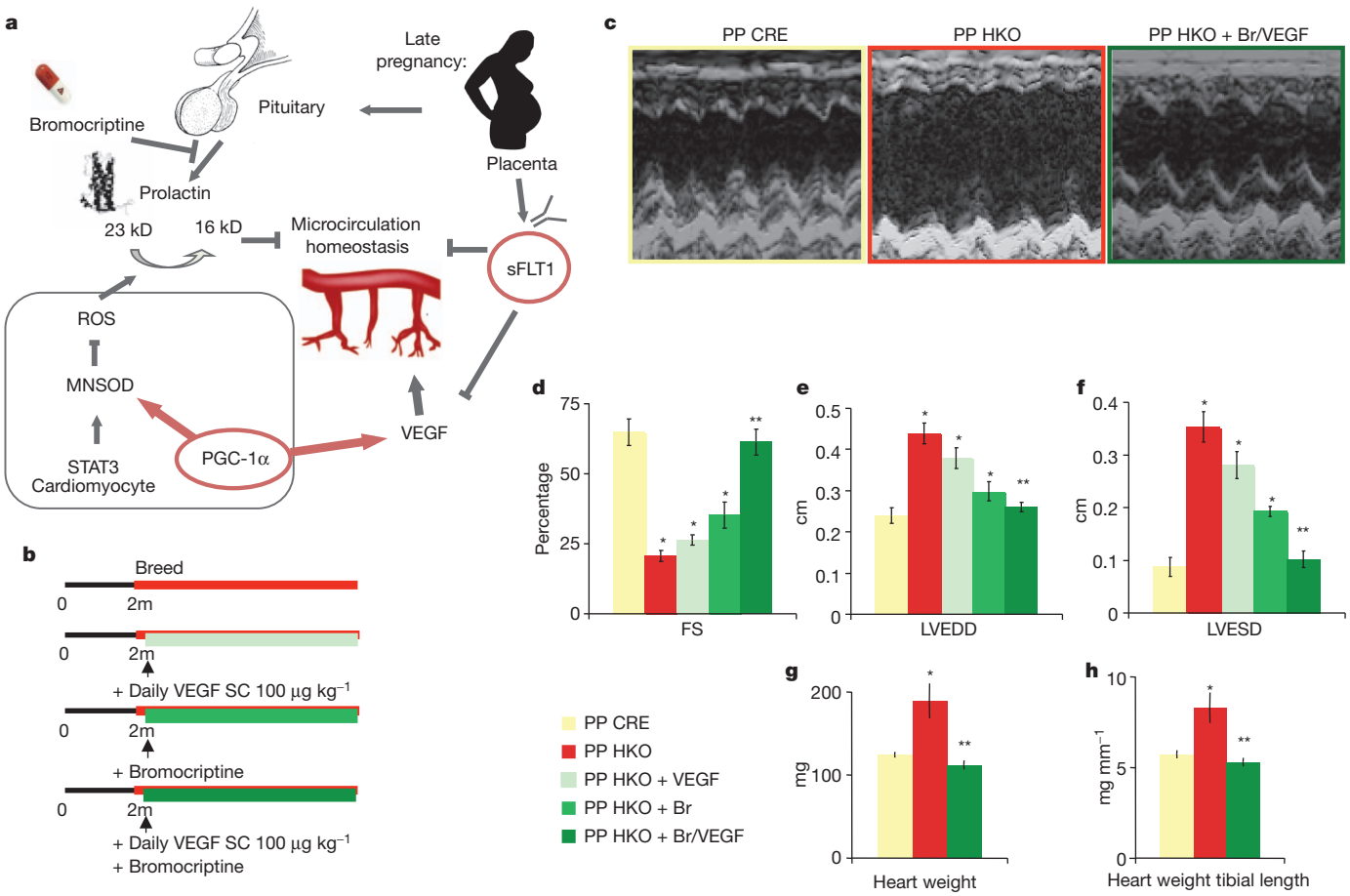


Figure 4 | Combined treatment with VEGF and bromocriptine rescues PPCM in PGC-1α HKO mice. **a**, Schema of the proposed role of cardiac PGC-1α in the regulation of cardiac angiogenesis, and in defending against pregnancy-induced anti-angiogenic factors. **b**, Experimental outline. m, months; SC, subcutaneous. **c**, Sample echocardiograms from PP HKO, PP CRE and PP HKO mice receiving both bromocriptine and VEGF treatments (PP HKO + Br/VEGF). **d–h**, Echocardiographic measures (**d–f**), heart weight (**g**) and heart weight:tibial length ratio (**h**), in PP mice of the indicated genotypes. $n \geq 5$ for all groups. Error bars are ± s.e.m. $^*P < 0.05$ versus PP CRE control. $^{**}P < 0.05$ versus PP HKO.

(Fig. 4c). After two pregnancies, heart weights and all echocardiographic indices of cardiac function (fractional shortening, LVEDD and LVESD) were normal in HKO mice (Fig. 4c–h). When only bromocriptine was used, some of the left ventricular dilation was prevented, but there was only minimal rescue of left ventricular function, showing that rescue of both pathways is necessary (Fig. 4c–h). Together, these data indicate that PPCM can be caused by an angiogenic imbalance and vascular dysfunction, at least in rodents.

Pre-eclampsia and cardiac dysfunction

To test the idea that anti-angiogenic signalling can cause cardiac dysfunction in pregnant women, we studied women with pre-eclampsia, in whom VEGF signalling is compromised owing to high serum levels of anti-angiogenic sFLT1[21] (see Supplementary Table 1 for patient characteristics). Cardiac function was evaluated non-invasively by measuring the myocardial performance index (MPI; also known as the Tei index) and other indices of cardiac function with cardiac echocardiography. MPI measures the relative duration of isovolaemic contraction and relaxation (Fig. 5a), and is a sensitive marker of diastolic function[22–24]. Women with pre-eclampsia had markedly increased serum levels of sFLT1 (Supplementary Fig. 5a, $P = 0.005$), as previously shown[21]. Notably, women with pre-eclampsia also had a markedly abnormal MPI (Fig. 5b and Supplementary Table 2, $P = 0.01$) and E/E' (Fig. 5c and Supplementary Table 2, $P = 0.02$), another sensitive measure of cardiac diastolic dysfunction that compares early diastolic mitral annulus (E') and transmitral (E) flow velocities[25]. Moreover, the MPI correlated with levels of circulating sFLT1 (Fig. 5d, $R = 0.59$,

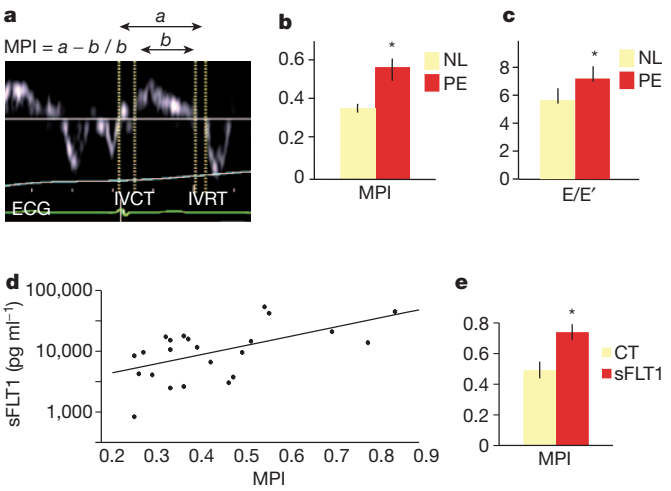


Figure 5 | Women with pre-eclampsia have depressed cardiac function that correlates with circulating sFLT1 levels, and sFLT1 causes cardiac dysfunction in mice. **a**, Sample tracing of echocardiographic tissue Doppler imaging. IVCT, isovolaemic contraction time; IVRT, isovolaemic relaxation time. **b**, **c**, Elevated MPI (**b**) and E/E' (**c**) in women with pre-eclampsia (PE) versus normal (NL) pregnancies. $P = 0.01$ and $P = 0.02$, respectively. **d**, Elevated MPI correlates with sFLT1 levels. $R = 0.59$. $P = 0.003$. **e**, Elevated MPI in pregnant mice infected with adenovirus expressing sFLT1. $P = 0.01$, $n \geq 5$ for all groups. $^*P < 0.05$ versus control.

$P = 0.003$). Elevated blood pressure in the pre-eclamptic women (Supplementary Table 1) is unlikely to explain the worsening MPI, because MPI is thought to reflect cardiac function independently of blood pressure[22], and pregnant women with similar mild elevations of blood pressure but without pre-eclampsia have normal cardiac function[26]. Instead, these data suggest that elevated sFLT1 causes the diastolic dysfunction. To test this idea directly, sFLT1 was delivered systemically to pregnant mice by intravenous injection of adenoviruses expressing sFLT1, and MPI was examined using high-resolution murine echocardiography. sFLT1 caused significant increases in MPI in these mice within 10 days (Fig. 5e). These data, taken together with published observations in patients receiving anti-angiogenic therapies[10,11], strongly suggest that elevated sFLT1 causes cardiac dysfunction in women with pre-eclampsia. Although the left ventricular dysfunction recovers following delivery in many patients, a second insult in some women probably precipitates PPCM.

sFLT1 causes cardiomyopathy and is high in PPCM

The above observations strongly support the idea that PPCM can be induced by excess anti-angiogenic signalling, including the high expression of sFLT1 during late gestation seen both in women[27] and mice (Supplementary Fig. 5c, $P = 0.009$). To test this idea directly, sFLT1 was delivered systemically to nulliparous mice, as above. PGC-1α HKO mice that received sFLT1 developed profound cardiac failure within 3 weeks; these mice had increased cardiac weight and marked decreases in fractional shortening on echocardiography (Fig. 6a–c). This was accompanied by a marked drop in vascular density (Supplementary Fig. 6), although not in larger vessels (Supplementary Fig. 7). Wild-type mice also showed significant, though less extensive, decreases in vascular density and cardiac function after exposure to 3 weeks of sFLT1. Thus, sFLT1 alone is sufficient, even in the absence of pregnancy, to cause dramatic cardiomyopathy in the setting of a heart that is unable to withstand the anti-angiogenic insult.

To investigate further whether elevated sFLT1 levels in humans could be contributing to PPCM, plasma from women with PPCM was acquired 4–6 weeks post-partum and sFLT1 levels were measured. sFLT1 levels usually return to normal within 48–72 h after delivery[28]. sFLT1 levels were elevated in a large subset of these PPCM patients ($P = 0.002$), remaining up to five- or tenfold higher than the levels in control participants (Fig. 6d). Post-partum sFLT1 levels can remain slightly higher in subjects with pre-eclampsia[29,30], but the levels found here are notably higher. Thus, the findings are consistent with the idea that a substantial percentage of PPCM subjects have been exposed to pre-eclampsia, and that secretion of sFLT1 persists inappropriately post-partum. Indeed, in our own institution, 33% of the last 75 cases of PPCM were associated with pre-eclampsia (Fig. 6e, f), markedly more than the population rate of 3–5% (ref. 31). The persisting extra-placental source of sFLT1 in the post-partum period is not known, and may include placental remnants, circulating mononuclear cells[32] or shed syncytial microparticles[33].

Discussion

Our study shows that angiogenic imbalance in the heart during the peri-partum period may lead to PPCM in mice and in humans. The data indicate that PPCM is caused by a 'two-hit' combination of, first, systemic anti-angiogenic signals during late pregnancy and, second, a host susceptibility marked by insufficient local pro-angiogenic defences in the heart. The first hit explains why PPCM is a disease of the late gestational period, which is precisely when circulating anti-angiogenic factors such as sFLT1 peak in pregnancy[21,34]. Other pathways, such as prolactin or excess angiotensin II signalling, may also be involved[3,35]. The first hit is also worse in pre-eclampsia, which is characterized by markedly elevated sFLT1 levels. Associations between pre-eclampsia and PPCM have been well documented in many populations[1,2,36–41](Supplementary Table 3). Interestingly, some studies involving women of African descent have not found an association between hypertensive disorders of pregnancy and PPCM[42], suggesting that there is ethnic variability in the pathogenesis of PPCM. It is also possible that PPCM with and without associated pre-eclampsia have different pathogeneses[43]. Overall, our data suggest that elevated sFLT1 levels in pre-eclampsia contribute to at least the PPCM that is associated with pre-eclampsia. We further propose that elevated sFLT1 levels in fact present a challenge to the myocardium in all pregnancies, thus explaining why the peri-partum period puts women at risk of developing heart failure, even in the absence of pre-eclampsia. Interestingly, other situations of elevated sFLT1 (twin pregnancies) and recurrent exposures to sFLT1 (multiple pregnancies) are also strong risk factors for PPCM even in the absence of pre-eclampsia[2,43,44].

Only a minority of women with pre-eclampsia develops PPCM, consistent with the existence of a second hit. Abnormal PGC-1α function is such an event in rodents, and it may also be a second hit in the case of humans. A number of previously identified processes may also constitute this second hit, including myocarditis, immune activation, viral infection and/or autoantibodies[43]. Interestingly, PGC-1α expression is repressed by inflammatory states in the heart and elsewhere[45,46], suggesting that many of the above processes that are implicated in PPCM may partly converge on PGC-1α. Consistent with this, we found repressed PGC-1α expression in cardiac samples from women with PPCM (Supplementary Fig. 8). Abnormal STAT3 function and ROS production[3] and genetic predispositions[47] may also be contributing factors.

In conclusion, the data presented here support the idea that PPCM is partly a two-hit vascular disease due to imbalances in angiogenic signalling, and that anti-angiogenic states such as pre-eclampsia or multiple gestation substantially worsen the severity of the disease. Our data may explain why pregnancy triggers PPCM, and also the long-standing epidemiological observation that pre-eclampsia is a risk factor for developing PPCM. Pro-angiogenic therapies such as exogenous VEGF121, or removal of sFLT1 itself[48], may therefore be beneficial in PPCM.

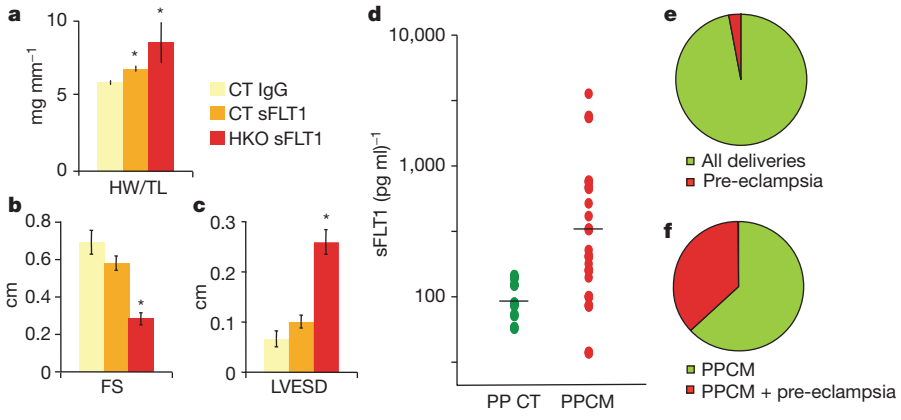


Figure 6 | sFLT1 is sufficient to induce cardiomyopathy in HKO mice, women with PPCM have elevated sFLT1 levels, and pre-eclampsia as a risk factor for PPCM. a–c, Heart weight:tibial length ratios (**a**), echocardiographic fractional shortening (**b**) and LVESD (**c**) in HKO mice injected with adenovirus expressing sFLT1, versus controls. **d**, Elevated sFLT1 levels in post-partum women with PPCM. $P = 0.002$. **e**, **f**, Prevalence of pre-eclampsia among all deliveries (**e**) and among women with PPCM (**f**) at Harvard teaching hospitals in the previous 9 years.

METHODS SUMMARY

All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee. Human soluble VEGF121 was a gift from Scios. Bromocriptine treatments were carried out as previously described[3]. Human studies were approved by the institutional review board of Beth Israel Deaconess Medical Center. Informed consent was obtained from all subjects. Angiogenic factor assays were performed with commercially available ELISA assays (R&D systems).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to Z.A. (zarany@bidmc.harvard.edu) or D.H.-K (hilfiker.denise@mh-hannover.de).

METHODS

Animal studies. All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee. Mice bearing floxed alleles of PGC-1α flanking exons 3 and 4, and mice containing the α-MHC::CRE transgene were gifts from B. Spiegelman[49] and M. Schneider[50], respectively. Mice were maintained on a standard rodent chow diet with 12-h light and dark cycles. For murine echocardiography, the chest hair was removed with a topical depilatory agent, and two-dimensional images were visualized using a Vivid FiVe echocardiography system (GE Medical Systems) on mice that were not anaesthetized. Parasternal short-axis projections were visualized and M-mode recordings at the mid-ventricular level were recorded. Heart rate, LVEDD and LVESD were measured in at least three beats from at least three recordings and averaged, and left ventricular fractional shortening was then calculated (fractional shortening = (LVEDD–LVESD)/LVEDD). For the high-resolution MPI studies, a VisualSonics 2100 echocardiography machine was used on mice anaesthetized with isoflurane, and the MPI was calculated using the manufacturer's software program. SPECT/CT imaging of mice was performed by the Longwood Area Small Animal Imaging Facility (SAIF). For the VEGF treatment studies, human VEGF121 (100 $\mu g\,kg^{-1}$) was injected subcutaneously daily, versus saline as the control. For the bromocriptine studies, bromocriptine was added to the drinking water. Mice were bred starting at the age of 8 weeks while receiving either bromocriptine in the drinking water or daily subcutaneous VEGF121, or both.

Cells and reagents. All reagents were from Sigma, unless otherwise indicated. Human soluble VEGF121 was a gift from Scios. Staining of capillaries was performed using anti-CD31 antibody (BD Pharmingen) or isolectin B4 (Vector Lab). Quantification of capillaries was performed computationally, using Volocity software (Improvision, PerkinElmer), on three random fields chosen from the septum of transverse sections from the mid-heart. Staining of arterioles was performed using anti-SMA antibody (Santa Cruz) and quantified similarly, using random low-power fields. All quantifications were performed blindly. Isolation and culture of primary NRVMs was performed as described. Cells were infected with adenovirus at a multiplicity of infection of 10× to 30×, and mRNA expression was measured 24 or 48 h later. The adenovirus expressing PGC-1α and sFLT1 have been described[51,52]. Prolactin, VEGF and sFLT1 ELISA assays were from R&D Systems. The thiobarbituic acid reactive substances (TBARS) assay was performed on cardiac extracts according to the manufacturer's instructions (Cayman).

Gene expression studies. Total RNAs were isolated from mouse tissue or cultured cells using the Trizol method (Invitrogen). Samples for real-time polymerase chain reaction (PCR) analyses were reverse transcribed (Invitrogen), and quantitative real-time PCR reactions were performed on the complementary DNAs in the presence of fluorescent dye (SYBR green) on a BioRad CFX 384 Touch real-time PCR detection system. DNA products of the expected size were confirmed for each primer pair.

Endothelial migration assay. NRVMs in 24-well plates were infected with adenovirus expressing GFP or PGC-1α for 34 h. bovine serum albumen (BSA) or sFLT1 (100 $ng\,ml^{-1}$) was added to the media for 12 h. Then, 5×10^4 cells of HUVECs at 5×10^4 were put on the upper compartment of transwells (8.0-μm pore size, Corning no. 3422) pre-warmed with EBM2 media overnight at 37 °C. HUVEC migration to the lower compartment of transwells was measured after 12 h. Migrated HUVECs were fixed with 4% paraformaldehyde in PBS for 20 min at 25 °C, cells remained on the upper compartment were removed with a cotton swab. Cells were blocked with 5% BSA in PBS 0.2% Tween (PBST) and stained with phallodin fluorescein isothiocyanate in PBST for 4 h to visualize filamentous actin. Transwell inserts were washed three times in PBST and mounted onto slides with 4′,6-diamidino-2-phenylindole (DAPI) mounting medium.

Human studies. The institutional review board of Beth Israel Deaconess Medical Center in Boston approved this study. Eligible women were enrolled after providing written informed consent from November 2009 to May 2010. Pregnant women at least 18 years of age with a singleton pregnancy of at least 24 weeks and less than 41 weeks, and either a diagnosis of pre-eclampsia or without any hypertensive disorder of pregnancy were eligible. Exclusion criteria included pre-existing cardiovascular disease, pulmonary disease and non-gestational diabetes mellitus. Participants were recruited after admission to labour and delivery, the ante-partum floor or during a routine prenatal visit. All clinical data were taken from medical records. The diagnosis of pre-eclampsia was based on the National High Blood Pressure Education Program Working Group definition, also endorsed by the American Congress of Obstetricians and Gynaecologists (ACOG). A maternal–fetal medicine specialist confirmed all diagnoses. Archived plasma samples from subjects with PPCM have been previously described[3]. Patients in both studies were predominantly Caucasian. Retrospective analyses of PPCM and pre-eclampsia in the Harvard teaching hospitals were performed using the Harvard Shared Health Research Information Network (SHRINE)[53], a de-identified repository of aggregate patient information.

Human echocardiography. Bedside transthoracic echocardiograms were performed using a Siemens X-300 (Mountainview) machine, by two expert echocardiographers using P5-1 Transducer. Images were obtained with the patient lying in the left lateral decubitus position and were reported according to the American Society of Echocardiography guidelines. Images were stored in a cine-loop format with three cardiac cycles of non-compressed data with electrocardiogram information. The echocardiographers performed a comprehensive examination, which included a complete two-dimensional and colour flow Doppler assessment of the left ventricle, right ventricle and intra-cardiac valves. Specifically: ejection fraction with visual quantitative estimation; trans-mitral pulse wave Doppler (E and A waves and deceleration time); Doppler tissue image (both medial and lateral mitral annulus were interrogated, and the final value of peak velocity of E' was calculated as the average of three velocities at each location); MPI, with the calculation performed off-line using a Siemens Syngo DICOM viewing station (Mountainview). The echocardiograms were de-identified before calculating MPI. Ejection fraction, MPI and E/E' ratios were calculated. Each image was analysed blind by one of two echocardiographers.

Angiogenic factor assays. Women consented to a blood draw at the time of the echocardiogram. All samples for the MPI study were collected in the ante-partum before the delivery, whereas samples in the PPCM study were collected 4–6 weeks post-partum. The samples were centrifuged at 1,900*g* for 8 min and plasma was collected and stored at −80° C. Samples were randomly ordered and analysed by a single person in a blind fashion. ELISA assays for sFLT1 were performed with commercially available kits (R&D systems). All assays were performed in duplicate and values were averaged. If >20% difference was observed between duplicate values, the samples were re-analysed.

Data and statistical analysis. SAS 9.2 (SAS institute) was used for data analysis. All tests were two sided, and *P* values of less than 0.05 were considered statistically significant. Data are presented as mean ± standard error, or median and inter-quartile ranges, as indicated. Comparisons were made using the two-tailed Student's *t*-test or the non-parametric Mann–Whitney test, as indicated.

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