Placental Growth Factor Regulates Cardiac Inflammation Through the Tissue Inhibitor of Metalloproteinases-3/Tumor Necrosis Factor-α—Converting Enzyme Axis

Crucial Role for Adaptive Cardiac Remodeling During Cardiac Pressure Overload

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Background—Heart failure is one of the leading causes of mortality and is primarily the final stage of several overload cardiomyopathies, preceded by an early adaptive hypertrophic response and characterized by coordinated cardiomyocyte growth, angiogenesis, and inflammation. Therefore, growth factors and cytokines have to be critically regulated during cardiac response to transverse aortic constriction. Interestingly, the dual properties of placental growth factor as an angiogenic factor and cytokine make it a candidate to participate in cardiac remodeling in response to hemodynamic overload.

Methods and Results—After transverse aortic constriction, placental growth factor knockout mice displayed a dysregulation of cardiac remodeling, negatively affecting muscle growth. Molecular insights underscored that this effect was ascribable mainly to a failure in the establishment of adequate inflammatory response owing to an impaired activity of tumor necrosis factor-α—converting enzyme. Interestingly, after transverse aortic constriction, placental growth factor knockout mice had strongly increased levels of tissue inhibitor of metalloproteinases-3, the main natural TACE inhibitor, thus indicating an unbalance of the tissue inhibitor of metalloproteinases-3/tumor necrosis factor-α—converting enzyme axis. Strikingly, when we used an in vivo RNA interference approach to reduce tissue inhibitor of metalloproteinases-3 levels in placental growth factor knockout mice during transverse aortic constriction, we obtained a complete phenotype rescue of early dilated cardiomyopathy.

Conclusions—Our results demonstrate that placental growth factor finely tunes a balanced regulation of the tissue inhibitor of metalloproteinases-3/tumor necrosis factor-α—converting enzyme axis and the consequent TNF-α activation in response to transverse aortic constriction, thus allowing the establishment of an inflammatory response necessary for adaptive cardiac remodeling. (Circulation. 2011;124:1337-1350.)

Key Words: heart failure ■ inflammation ■ growth substances ■ ventricular remodeling ■ RNA, small interfering

Several cardiovascular diseases, such as aortic stenosis, hypertension, and myocardial infarction, impose a hemodynamic overload on cardiac walls, activating complex biological responses that culminate in tissue remodeling. Tissue remodeling initially starts as compensatory left ventricular (LV) hypertrophy (LVH), but eventually evolves in maladaptive remodeling, triggering the transition to heart failure.1,2

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An accurate intertwining of muscle growth, inflammation, and angiogenesis is pivotal to ensure the adaptive hypertrophic remodeling, and alterations of this equilibrium cause the deterioration of cardiac structure and function. Among the various molecular factors participating in the remodeling of...
the LV chamber in response to hemodynamic overload, a main role has been also attributed to the secretion of several cytokines and growth factors. For example, it has been shown that cytokines typically upregulated during transverse aortic constriction (TAC), such as interleukin-6 and tumor necrosis factor-α (TNF-α), have key roles in cardiac hypertrophy and protective effects during the maladaptive transition to heart failure.

Additionally, among the growth factors, the angiogenic ones seem to play a not trivial supportive role, besides inducing vessel growth, by contributing to the adaptation of cardiac muscle to hemodynamic overload. For example, defects in cardiac vascular endothelial growth factor (VEGF) impair the adaptive remodeling in overloaded hearts, accelerating the transition to heart failure.

Interestingly, the impact of a VEGF-related homolog, placental growth factor (PIGF), which shares properties of both angiogenic factor and cytokine, on cardiac remodeling induced by hemodynamic overload is still poorly explored. Expressed in cardiac tissue, PIGF increases in hypoxic/ischemic myocardium, contributes to pathological angiogenesis, such as vascular regeneration under tissue ischemia, and may have both positive or negative effects on tumor growth, suggesting that its environment can influence its effects. On the other hand, it has been demonstrated that the cytokine face of PIGF acts as a chemotactic agent for monocytes, and it is well known that monocytes participate in the cardiac inflammatory response to pressure overload and support adaptive cardiac remodeling. Moreover, TNF-α activation, one of the earliest inflammatory events in overloaded hearts, has recently been linked to PIGF to recruit myelomonocytic cells in inflamed tissues. In fact, TNF-α needs to be shed from a membrane-bound form by TNF-α–converting enzyme (TACE), and the main natural inhibitor of TACE is tissue inhibitor of metalloproteinases-3 (TIMP-3), the most abundant TIMP expressed in cardiac tissue. Interestingly, the lack of TIMP-3, producing excessive TNF-α activation, promotes maladaptive cardiac remodeling, favoring the transition to heart failure. On the other hand, anti–TNF-α therapy fails to be successful for heart failure treatment, thus suggesting that, even if excessive inflammatory responses are detrimental, impaired inflammation also could be harmful, hampering an adequate tissue response to injury. Thus, PIGF has several features that could make it a candidate to play a role in the complex cytoarchitectural response of the heart to hemodynamic overload.

In this study, we explored the pathophysiological role of PIGF in cardiac remodeling to pressure overload as both an angiogenic factor and a cytokine. We clarify that PIGF is increased in overloaded hearts and that, in this context, it influences mainly vessel dimensions. More important, we identify that PIGF finely tunes a balanced regulation of the TIMP-3/TACE axis and the consequent TNF-α activation in response to TAC, thus ensuring an adequate inflammatory milieu for adaptive cardiac hypertrophic remodeling.

### Methods

The detailed methodology is provided in the online-only Data Supplement.

### Animals and Surgery

Experiments were conducted according to European Communities Council Directive No. 86/609/EEC in 12- to 15-week-old C57BL/6J and PIGF knockout (KO)/wild-type (WT) male mice. Transverse aortic constriction was obtained as previously described.

### Drug Treatment and siRNA Studies

TAPI-1 (Peptides International) was administered daily by subcutaneous injection. Negative control, TIMP-3, and FITC-conjugated siRNA were synthesized by Santa Cruz and systemically injected. Recombinant PIGF (1.5 μg/kg body weight; R&D Systems) was administered daily by intraperitoneal injection starting 2 days before TAC.

### Echocardiographic Analysis

Echocardiographic analysis was performed with Vevo770 and Vevo2100 (Visualsonics Inc).

### Immunohistochemistry, Histology, and In Situ Hybridization

For immunohistochemical, histological, and in situ hybridization analyses, hearts were removed after diastolic arrest, postfixed, and cryosectioned.

### RNA, Reverse Transcription, Quantitative Polymerase Chain Reaction, and Polymerase Chain Reaction Array

TRizol reagent for RNA extraction and Superscript III kit (Invitrogen, Eugene, OR) for cDNA preparing, SYBR green polymerase chain reaction master mix, and mouse polymerase chain reaction Array (PAMM-013, SABiosciences) were used according to the manufacturers’ instructions.

### Western Blotting and Gelatin Zymography

Heart proteins were extracted and resolved by SDS-PAGE, followed by Western blotting or gelatin zymography, as described in the online-only Data Supplement.

### Tumor Necrosis Factor-α and Tumor Necrosis Factor-α–Converting Enzyme Activity

The TNF-α levels and TACE activity were measured with a high-sensitivity ELISA kit (Endogen, Pierce) and an α-secretase activity kit (Anspec), respectively.

### Statistical Analysis

Echocardiographic analyses were tested with repeated measures 2-way ANOVA and the Bonferroni post hoc test; data are presented as mean±SEM. Statistical significance of nonrepeated measures was calculated with 1-way ANOVA and the Bonferroni post hoc test when the sample size was ≥6 (data presented as mean±SEM) and with the Kruskal-Wallis test with Dunn post hoc tests for sample sizes <6 (data presented as actual points). For 2-group comparisons, the Mann-Whitney test was applied. Statistical analyses were performed with GraphPad software PRISM5 (GraphPad Software Inc, La Jolla, CA).

### Results

**Placental Growth Factor Is Upregulated During Transverse Aortic Constriction, and Its Absence Accelerates Transition Toward Dilated Cardiomyopathy**

Here, we show a time-dependent increase in cardiac PIGF in C57BL/6J mice exposed to TAC for 1 day and 1 week (Figure 1A). To explore the role of PIGF activation in pressure overload, we used echocardiography to follow up over time (1 to 2 to 4 weeks) PIGF KO and WT mice subjected to TAC.
The degree of aortic impedance imposed, ie, the trans-stenotic gradient, was similar in both genotypes (Table I in the online-only Data Supplement). Notably, although WT developed a typical concentric hypertrophy, as evidenced by increased relative wall thickness (Figure 1B and Table I in the online-only Data Supplement) and reduced LV internal diameter (Figure 1C and Table I in the online-only Data Supplement), PlGF KO displayed impaired hypertrophy and early dilated cardiomyopathy (Figure 1B and 1C and Table I in the online-only Data Supplement). In addition, cardiac fractional shortening (fractional shortening) in PlGF KO was significantly decreased at 1-week TAC compared with WT, with a further significant decline over time (Figure 1D and Table I in the online-only Data Supplement).

To better characterize the myocardial performance of KO compared with WT, another group of mice were followed up for a longer period of time (until 6 weeks). Further deterioration of cardiac function and structure was observed in KO, as shown by both conventional (Table II in the online-only Data Supplement) and speckle-tracking–based strain echocardiography (Table III in the online-only Data Supplement) along the longitudinal and radial axes (Figure 1E and 1F). In particular, myocardial tissue deformation, evaluated as the percent change in both strain and strain rate values of TAC versus sham, was significantly reduced in KO (Figure 1E and 1F). Displacement and velocity were also significantly reduced in KO after TAC (Figure 1E and 1F).
At 6 weeks from TAC, KO showed an increased mortality compared with WT, which was further aggravated at 8 weeks (Figure IA in the online-only Data Supplement). According to the phenotype of dilated cardiomyopathy and heart failure, shown by echocardiographic analysis, serum troponin I (Figure IB in the online-only Data Supplement), LV (Figure IC in the online-only Data Supplement), and lung (Figure ID in the online-only Data Supplement) weights were increased in KO compared with WT.

**Placental Growth Factor Knockout Mice Show Impaired Cardiomyocyte Growth and Increased Vessel Dimensions in Response to Transverse Aortic Constriction**

We looked at the response of cardiomyocytes and vessels, 2 major components of TAC-induced cardiac remodeling. Histological analysis of 1-week TAC indicated that KO displayed a defect in the typical cardiomyocyte growth showed by WT (Figure 2A and 2B). Further supportive of impaired hypertrophy, Akt phosphorylation, a hypertrophic signaling pathway usually induced by TAC, was markedly reduced in KO compared with WT (Figure IIA in the online-only Data Supplement). Moreover, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling analysis demonstrated an increase in apoptosis in KO (Figure 2D and 2E).

Because cardiomyocytes express PlGF receptor, VEGF receptor-1 (VEGFR-1), we explored possible direct effects of PlGF on cardiomyocyte growth by stimulating isolated cardiomyocytes with increasing doses of recombinant PlGF. No direct evidence of hypertrophic growth, elicited by PlGF, was detected (Figure IIB in the online-only Data Supplement), thus suggesting that the impaired hypertrophic response in overloaded KO hearts could not be ascribed to PlGF direct effects on muscle growth.

In terms of capillary status, overloaded WT LV showed a reduction in myocardial microvascular density (Figure 2F and 2G), as indicated by the absolute number of vessels per area, but the capillary/myocyte ratio (Figure 2A and 2C) was in turn increased. However, although KO displayed a reduction in the capillary density in the myocardium (Figure 2F and 2G) comparable to that of WT, their capillary/myocyte ratio in response to TAC remained unchanged (Figure 2A and 2C). Interestingly, KO showed a significant increase in vessel dimensions (Figure 2F), as evidenced by vessel area quantification (Figure 2H). To better characterize the impact of this particular vascular remodeling in KO during TAC, we performed an ultrasonographic tissue perfusion analysis by using microbubble contrast agent. We preserved KO by decreasing the LV myocardial flow observed after 1 week of TAC in WT (Figure IIC in the online-only Data Supplement), indicating that the lack of PlGF, increasing microvessel dimensions, compensates for the microvascular rarefaction induced by TAC. Strikingly, although 1-week TAC WT showed activated hypoxia-inducible factor-1α (Figure IID and IIE in the online-only Data Supplement), a typical signaling recruited by ischemic conditions, and accumulated hypoxic metabolites, as evidenced by hypoxprobe staining (Figure IIE in the online-only Data Supplement), KO mice did not display activation of any of them, thus suggesting that their microvascular remodeling preserves them from the typical myocardial ischemia accompanying TAC. More important, this particular vascular phenotype of KO cannot obviously explain their accelerated transition to heart failure.

**Absence of Left Ventricular Fibrosis and Impaired Inflammation in Placental Growth Factor Knockout Mice in Response to Transverse Aortic Constriction**

We then evaluated the effects of PI GF genetic ablation on myocardial fibrosis, finding that, although the Picrosirius Red–positive area in WT hearts increased in 1-week TAC, it was not significantly higher than baseline in KO (Figure 3A and 3B). Furthermore, transcription of collagen-I, collagen-III, and fibronectin, 3 extracellular matrix components typically overexpressed during TAC, was strongly increased in WT but not in KO (Figure 3C through 3E).

Myocardial fibrosis is due in large part to the inflammatory response established during TAC; interestingly, PlGF is at the same time a cytokine with attractive properties for myelomonocytic cells and a permeability factor. We found that lack of PlGF impaired the activation of vessel permeability induced by TAC in WT (Figure 1A) and negatively affected recruitment of CD68-positive monocytes in response to TAC (Figure 4B).

Accordingly, the expression pattern of typical proinflammatory and antiinflammatory mediators (interleukin-1α, interleukin-1β, TNF-α and transforming growth factor-β) was completely altered in a time-dependent fashion in the absence of PlGF (Figure 4C through 4F). In particular, the lack of PI GF abolished interleukin-1α (Figure 4C), TNF-α (Figure 4E), and transforming growth factor-β (Figure 4F) during the first early wave (6 hours) of upregulation induced by TAC and normalized after 3 days. Interestingly, 1 week after TAC, these cytokines showed a second wave of increase (Figure 4C, 4E, and 4F), accompanied by interleukin-1β upregulation (Figure 4D) in WT, whereas a still impaired response was observed in KO (Figure 4C through 4F). Overall, these results indicate that KO failed to establish an early inflammatory response to TAC and to control it over time.

Matrix metalloproteinases (MMPs) and their inhibitors are known to orchestrate the onset of inflammation during a tissue challenge by regulating cytokine bioavailability in the microenvironment. Thus, the cardiac expression profile of selected extracellular matrix structural constituents, proteases and protease inhibitors, was performed with a polymerase chain reaction array. Only TIMP-3 was significantly upregulated in KO but not in WT after TAC. On the other hand, among the MMP genes evaluated, MMP-10, MMP -7, and MMP -8 expression increased only in WT but not in KO overloaded hearts (Table IV in the online-only Data Supplement).

**Cardiac Tumor Necrosis Factor-α–Converting Enzyme Activity Is Induced by Placental Growth Factor and Is Involved in Muscular and Vascular Remodeling in Response to Transverse Aortic Constriction**

Among the inflammatory mediators analyzed, a key role in cardiac remodeling during TAC is played by TNF-α, and one
Figure 2. Impaired cardiomyocyte growth and increased vessel dimensions in placental growth factor (PlGF) knockout (KO) in response to transverse aortic constriction (TAC). A, Representative photomicrographs of cardiac sections from 1-week TAC/sham KO and wild type (WT) stained with wheat germ agglutinin and isoelectin and counterstained with DAPI. Scale bar=20 μm. B and C, Quantification of cardiomyocytes area (*P<0.01 vs sham) and vessels-to-cardiomyocytes ratio (*P<0.05 vs sham) in n=4 for each group. D and E, Representative photomicrographs of cardiac sections from 1-week TAC KO and WT stained with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling for apoptosis (green) and vinculin (red) and counterstained with DAPI (scale bar 50 μm) and relative quantification (n=4/group; *P<0.05 vs TAC WT). F, Representative photomicrographs of cardiac sections from 1-week TAC/sham KO and WT stained with isoelectin. Scale bar=100 μm. G and H, Quantification of vessel number (G; *P<0.01 vs sham) and area (H; *P<0.05 vs sham) in n=4 per group.
of the main regulators of extracellular matrix proteases activation, TACE, is critically required for the occurrence of TNF-α release in its active soluble form.

To better clarify the PlGF effect on TNF-α, we measured the amount of active soluble TNF-α early after TAC (at 1 day) and then at 1 week. We found that KO had TNF-α levels almost equal to those of sham, whereas WT showed an early and consistent TNF-α increase (Figure 5A). Then, we evaluated TACE activity, finding that in WT TAC was able to rapidly activate TACE (at 1 day) and maintain its activation over time (1 week) (Figure 5B). Conversely, KO mice were unable to induce TACE (Figure 5B), thus indicating that PlGF is able and indispensable to condition TACE activation. Interestingly, double staining for TACE and the main cellular types involved in cardiac remodeling revealed that resident cells like cardiomyocytes (Figure 5C) and vessels (Figure 5D) and inflammatory cells (CD68-positive monocytes; Figure 5D) activated TACE in response to pressure overload.

Recruitment of the TACE pathway also induces a process of regulated intramembrane proteolysis, affecting a wide variety of transmembrane proteins.23 Interestingly, analysis of membrane and soluble fractions from overloaded heart homogenates revealed the processing of VEGFR-1, forming the 2 soluble fragments, a cytosolic 80-kDa fragment and an extracellular 120-kDa fragment, only in WT but not in KO (Figure 5C). Similarly, KO failed to activate the TACE-induced Notch-1 pathway, hampering NICD formation (Figure 5D) and the consequent upregulation of the transcription factor Hes-1 (Figure 5E). Therefore, KO showed an impaired proteolytic processing that was dependent on TACE activity, further supporting that PlGF is crucial to permit the cascade of molecular events leading to TACE activation in response to TAC. Further supporting a failure in activating TACE pathway, KO did not show TAC-induced activity of MMP-2, a typical MMP downstream of TACE, as evidenced by gelatin zymographic analysis (Figure 5F).

Finally, we treated WT during TAC with TAPI-I, an inhibitor of TACE activity, to see whether this strategy reproduced the phenotypic traits displayed by KO. Noticeably, we found a cardiac response to TAC similar to that previously observed in KO. In particular, echocardiographic analysis revealed impaired hypertrophic response (as shown by relative wall thickness and LV internal diameter in Figure 6A and 6B) and cardiac function (fractional shortening; Figure 6C), accompanied by increased vessel dimensions (Figure 6D and 6E).

Figure 3. Impairment of left ventricular (LV) fibrosis in placental growth factor (PlGF) knockout (KO) in response to transverse aortic constriction (TAC). A and B, Picrosirius Red staining (A) and quantification of positive area (B) of cardiac sections from 1-week TAC/sham KO and wild type (WT). Scale bar=200 μm (1 mm for the inset) (n=4 per group; *P<0.05 vs sham). C through E, mRNA expression levels of collagen-I, collagen-III, and fibronectin in 1-week TAC/sham KO and WT (n=4 per group; *P<0.05 vs sham).
reported that TIMP-3 is upregulated by TAC later, i.e., at 3 weeks, probably to dampen the ongoing inflammatory response. Our unexpected result suggested that PlGF could have a role in maintaining normal TIMP-3 expression levels, known to be important in balancing TACE activation. To prove that PlGF directly regulates TIMP-3 expression, we treated PlGF KO mice with recombinant PlGF. As shown in Figure 7B, PlGF treatment was able to restore TIMP-3 expression to normal levels during TAC, thus demonstrating a direct dependence of TIMP-3 on PlGF.
Figure 5. Placental growth factor (PIGF) controls tumor necrosis factor-α (TNF-α) converting enzyme (TACE) activation during transverse aortic constriction (TAC). A and B, The TNF-α levels (*P<0.01 vs sham) and TACE activity (*P<0.01 vs sham; #P<0.01 vs TAC wild type [WT] of the same time point) in TAC/sham (n=4 per group). C through E, Representative photomicrographs of cardiac sections from TAC/sham KO and WT mice stained for TACE and (C) α-actinin, (D) isoelectin, and (E) CD68 and counterstained with DAPI. Scale bar=20 μm. F, Vascular endothelial growth factor receptor -1 (VEGFR-1) proteolytic processing in 1-week TAC/sham: C term, 210-kDa full-length receptor in membrane fraction; N term, 120-kDa extracellular fragment in soluble fraction; C term, 80-kDa intracellular fragment in soluble fraction (n=3). G, Notch-1 activation after NICD intracellular fragment formation in 1-week TAC/sham animals (n=3). H, mRNA expression levels of Hes-1 transcription factor in 1-week TAC/sham (n=4 per group; *P<0.001 vs TAC WT). I, Gelatin zymography for matrix metalloproteinase-2 (MMP-2) activity in 1-week TAC/sham.
Because TIMP-3 is a protein secreted in the extracellular matrix and its regulation is mainly at the transcriptional level, we used the in situ hybridization technique to confirm and localize the increased expression of TIMP-3 at the mRNA level. As shown in Figure 7C, TAC induced an increased staining for TIMP-3 mRNA in KO compared with WT. Both cardiomyocytes (red arrow) and vessels (red arrowheads) were intensely stained (Figure 7C).

Thus, we looked for a strategy to normalize early TIMP-3 overexpression in KO by interfering at transcriptional level. To this aim, we used an in vivo silencing approach by systematically administering a small interfering RNA (siRNA) in KO. We first proved the efficacy of this approach in our model by administrating a control siRNA-FITC to visualize its delivery in cardiac tissue by histology (Figure 7D).

We started siRNA administration for both TIMP-3 and negative control 3 days before TAC, repeating the treatment every 5 days. Western blot analysis revealed that only siRNA for TIMP-3 was able to reduce the cardiac TIMP-3 protein levels of KO, starting at 1-day TAC (Figure IIIA in the online-only Data Supplement). The effect was even more marked at 1-week TAC (Figure 7E and Figure IIIA in the online-only Data Supplement). Therefore, this siRNA-based strategy allowed a reduction in the abnormal TIMP-3 protein levels observed in KO in response to TAC (Figure 7E) and a restored activation of TACE (Figure 7F) without compensating by upregulating the other inhibitors of ADAMs/MMPs like TIMP-1 and TIMP-2 (Figure IIIB in the online-only Data Supplement).

Strikingly, TIMP-3 siRNA KO developed an adaptive hypertrophic remodeling (as shown by relative wall thickness and LV internal diameter in Figure 8A and 8B) and retained cardiac function (fractional shortening; Figure 8C) in response to TAC, whereas negative control KO still displayed eccentric hypertrophic remodeling (Figure 8A and 8B) and cardiac dysfunction (Figure 8C) after TAC.

At histological evaluation, wheat germ agglutinin and isolectin staining revealed that TIMP-3 siRNA rescued TAC-induced cardiomyocyte growth (Figure 8D and 8E) and restored vessel dimensions (Figure 8D and 8F) in KO compared with negative control siRNA. Finally, the CD68-positive staining found in TIMP-3 siRNA KO also indicated a rescue of the inflammatory component in response to TAC (Figure 8G). Together, these results demonstrate that targeting TIMP-3 levels rescues the inflammatory adaptive response to hemodynamic overload, allowing a proper LV myocyte and vascular remodeling in KO.

**Discussion**

In this study, we identify PIGF as an indispensable component of the cardiac response to pressure overload because its ablation hampered the development of typical compensatory hypertrophy, hastening the transition toward dilated cardiomyopathy and heart failure. In particular, the lack of PIGF induced an impaired cardiomyocyte growth with increased vessel dimensions. These features of cardiac remodeling in KO were accompanied by an impaired inflammatory response and the development of LV fibrosis.
molecular mechanisms highlighted a regulating role of PlGF on TACE, a key molecule of tissue inflammatory response recently identified as a master regulator in cardiac hypertrophic remodeling. We found that PlGF is necessary to maintain normal levels of cardiac TIMP-3, the TACE natural inhibitor, allowing early TACE activation and consequently TNF-α production during TAC. Strikingly, the decrease in TIMP-3 levels with an siRNA approach rescued early dilated cardiomyopathy and cardiac dysfunction.

The deterioration of cardiac structure and function displayed by KO in response to hemodynamic overload was demonstrated by conventional and strain-based echocardiographic methods.
A graphic analysis, clearly indicating a worse global cardiac function of KO. Moreover, histological examination confirmed an impaired adaptive hypertrophic response in KO, accompanied by increased cardiomyocyte apoptosis.

Overall, this phenotype seems to recapitulate traits observed during inhibition of other angiogenic factors like endogenous VEGF. In particular, VEGF sequestering with Flk-1 ectodomain similarly impaired adaptive cardiac hypertrophy with accelerated transition to heart failure. In this case, the impairment of cardiac hypertrophic response was due to a marked reduction in coronary capillary density. On the contrary, our data indicate that, even if the absence of PlGF hampers compensatory LVH response to TAC, this phenotype should be ascribed to factors other than capillary rarefaction. Actually, KO mice display a vascular remodeling with greater capillary dimensions than those observed in WT mice. Such a condition ensures an improved myocardial perfusion with greater capillary dimensions than those observed in WT mice. Moreover, TAC-induced myocardial interstitial fibrosis, markedly increased in VEGF deprivation, was conversely reduced in KO, suggesting that the deterioration of cardiac structure and function developed by overloaded KO has to be ascribed mainly to a defect in adaptive muscle growth, because both myocardial perfusion and interstitial...
fibrosis responses could have been traits leading to a more favorable fate. However, our analysis with recombinant PlGF excluded a direct effect of PlGF on cardiomyocyte growth, suggesting that the adverse cardiac remodeling in KO should recruit other mechanisms.

On this topic, it is noteworthy to emphasize that KO markedly showed reduced monocyte recruitment in overloaded hearts and failed to upregulate expression of typical inflammatory cytokines. Furthermore, during TAC, the lack of PlGF impaired the cardiac release of active TNF-α, a cytokine with a great impact on LV remodeling recently associated with the effect of PlGF on myelomonocytic cell recruitment to the inflamed tissue. This picture is strongly supportive of a key role of PlGF in establishing an adequate and necessary inflammatory response to TAC. The reduced TNF-α levels in KO were dependent on an impaired activity of TACE, a pivotal regulator of TNF-α release and newly identified player in cardiac remodeling resulting from TAC.

Besides TNF-α, TACE activation is critically required for the occurrence of cleavage of other proteolytic substrates. As further support of the role of PlGF in activating TACE during TAC, we found that other shedding products were formed only in WT and not in KO. Among them, we proposed 2 proteins that could be potentially relevant during TAC. One is sVEGFR-1, derived by the full-length receptor of PlGF (VEGFR-1); the other is Notch-1, which, after shedding, forms an intracellular fragment called NICD with transcriptional functions. Interestingly, it has recently been demonstrated that a VEGFR-1 shedding product can have antiangiogenic properties at least in vitro, in accordance with our finding that inhibition of PlGF and thus of its receptor processing has growth function on microvasculature. In addition, well-established knowledge clearly shows that Notch-1 inhibition has proangiogenic properties. On the whole, this strongly points to an important upstream role of PlGF-induced TACE activation during cardiac response to TAC. Furthermore, our results that pharmacological TACE inhibition displayed impaired LV remodeling and increased vessel dimensions in response to TAC, resembling those previously observed in KO, strongly support that PlGF regulates tissue remodeling by controlling TACE activity.

Typically, the process involving the proteolytic cleavage of substrates is a noncell autonomous mechanism, always requiring the contact of a cell presenting the substrate and another with the protease (TACE in this case). The involvement of a noncell autonomous mechanism is further supported by the evidence that TACE was localized both in resident cells such as cardiomyocytes and vessels and in inflammatory cells. Thus, because VEGFR-1 is expressed by resident cardiac cells like endothelium and cardiomyocytes and circulating cells like monocytes, PlGF could orchestrate the complex cardiac remodeling by acting on all of these cell types. Interestingly, we identified that expression of TIMP-3, the only extracellular matrix–bound TIMP known to check inflammation through inhibition of TACE with recognized functions during TAC-induced cardiac remodeling, was significantly increased in KO after TAC, whereas it remained unchanged in WT. With in situ hybridization, we found that the increased mRNA expression was present mainly in vessels and cardiomyocytes, both known to express PlGF receptor and involved in the maladaptive remodeling observed in KO.

Strikingly, when we used an siRNA approach to reduce TIMP-3 expression to normal levels during TAC in KO, we found a complete phenotype rescue at echocardiographic analysis, hampering the early transition toward dilated cardiomyopathy. The histological examination revealed a rescue of cardiac hypertrophy and monocyte recruitment, as well as a restoration of vessel dimensions. The reactivation of TACE in KO treated with TIMP-3 siRNA suggests that TIMP-3 is the primary target of PlGF. Therefore, it emerges that the TIMP-3/TACE axis must be tightly balanced during an inflammatory response and in cardiac TAC. Only this balance allows the establishment of an LVH response and correct matrix remodeling on TAC, both indispensable to compensate for stress on cardiac walls and to delay the transition to heart failure.

On this topic, it is noteworthy to mention that Kassiri et al demonstrated that TAC is able to induce transcriptional regulation of TIMP-3 but only after 3 weeks after TAC, likely to check the already activated inflammatory response. Here, we show that in the absence of PlGF, the increased expression of TIMP-3 turns out to be an earlier event, hampering the establishment of inflammatory response. Thus, during TAC, PlGF is crucial to retain balanced cardiac TIMP-3 levels.

Our results seem to conflict with previous data showing that TIMP-3 KO, obtained by embryonic knockdown of the gene, led to natural deterioration of cardiac structure and function with aging and accelerated transition to heart failure on TAC. These effects were ascribed mainly to a dysregulated TACE activation and excessive cytokine production, leading to exacerbated inflammation, fibrosis, and hypertrophy and recognizing that maladaptive matrix remodeling promotes abnormal cardiac structure and function. However, our approach, based on in vivo siRNA, was able to target TIMP-3 expression in a very specific time window, thus probably avoiding the deleterious effect of a complete lack of this important regulator of tissue remodeling. Our results demonstrate that the establishment of an early balanced inflammatory response during TAC is necessary for a compensatory hypertrophic response, retaining cardiac function.

Finally, it is of note in the upcoming evidence that, besides the already-mentioned downstream targets of the TIMP-3/TACE axis, some specific MMPs have recently been identified as being involved in the development of compensatory LVH response to TAC. Among them, we found that typical TAC-induced MMP-2 activation was impaired in KO.

Taken together, our results and those from previous studies demonstrate that the balance between TIMP-3 and TACE is pivotal in LV response to injury and provide insight into novel therapeutic strategies that should be taken into account to limit adverse remodeling during the onset of heart failure.

A potential limitation of our study is the use of a germline KO model that does not allow a temporal and/or spatial selectivity of intervention on PlGF expression. Selective ablation of PlGF in each of the cell types involved would allow us to clearly distinguish the contribution of PlGF...
signaling in cardiomyocytes, endothelium, and monocytes. Work is underway on this issue.

Conclusions
We identify a novel function of PlGF as a regulator of TIMP-3/TACE axis during cardiac remodeling in response to TAC. In particular, our data support a role for PlGF in exerting a transcriptional regulation of TIMP-3 to tightly check inflammation.

This finding arouses important questions of potential clinical remark. One interesting point is about the clinical impact of therapeutic applications of anti-PlGF antibodies. Clinical studies have indeed shown that PlGF levels correlate with poor prognosis in various cancer forms, and consequently, anti-PlGF monoclonal antibodies have been developed as a strategy to slow tumor growth.9,10 Besides the discrepancies among different evidence reported about the efficacy of tumor therapies exploiting anti-PlGF antibodies,9,10 this novel role of PlGF in cardiac remodeling makes further consideration necessary when deciding on this therapy in patients with increased cardiovascular risk.

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Disclosures
None.

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CLINICAL PERSPECTIVE

Our results highlight a novel role for placental growth factor (PIGF) in cardiac remodeling to pressure overload by orchestrating the inflammatory response. Important questions of potential clinical importance ensue from these findings. First, the proposed role for PIGF in protection from the development of dilated cardiomyopathy strongly suggests that a pharmacological therapy with recombinant PIGF could be exploited in clinical practice as a promising treatment for heart failure. This will allow maintenance of a balanced positive inflammatory response, necessary to cope with cardiac remodeling in challenging conditions. Another remarkable point that emerges from our findings concerns the clinical impact of antitumor therapies exploiting anti-PIGF antibodies. The conflicting results showed that such clinical studies should now also take into account our study showing a novel role for PIGF in cardiac remodeling and further consideration when deciding on a therapy with anti-PIGF antibodies in patients with increased cardiovascular risk. Finally, the potential importance of PIGF effects on cardiac remodeling in relation to peripartum dilated cardiomyopathy should be considered. Indeed, this condition is associated with different risk factors, notably hypertension and low levels of circulating PIGF. Our model of pressure overload in PIGF-deficient mice may resemble such a situation and suggest a possible reason why pregnancy may lead to dilated cardiomyopathy when concomitant with the presence of risk factors such as hypertension and low levels of PIGF.
Placental Growth Factor Regulates Cardiac Inflammation Through the Tissue Inhibitor of Metalloproteinases-3/Tumor Necrosis Factor-α-Converting Enzyme Axis: Crucial Role for Adaptive Cardiac Remodeling During Cardiac Pressure Overload
Daniela Carnevale, Giuseppe Cifelli, Giada Mascio, Michele Madonna, Mauro Sbroggiò, Cinzia Perrino, Maria Grazia Persico, Giacomo Frati and Giuseppe Lembo

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals and surgery

Experiments were conducted according to European Communities Council Directive No. 86/609/EEC. 12-15 weeks old C57Bl/6J and PlGF KO/WT male mice were kept under a constant 12-h light-dark cycle at 22-25 °C with standard chow and water provided ad libitum. Transverse aortic coarctation (TAC) was obtained in mice anesthetized with ketamine/xylazine, performing coarctation between truncus anonymous and left carotid, as previously described (1). Sham mice underwent the same surgical procedure without realizing stenosis. A systolic trans-stenotic gradient was measured by echo Doppler (Vevo 770, Visualsonics inc., Canada).

Drug treatment and siRNA Studies

TAPI-I \([N-R-(2-hydroxyaminocarbony)methyl]-4-methylpentanoyl-L-naphthylanyl-L-alanine\) 2 aminoethyl amile-I (Peptides International) was dissolved in 0.5% Acetic Acid/1% NaCl and daily administered by subcutaneous injections (2mg/kg/die) as previously demonstrated to inhibit TACE activity (2). Treatment was started three days before performing TAC/sham surgery.

TIMP-3 expression knockdown was obtained using a small-interfering RNA (TIMP-3 siRNA), synthesized by Santa Cruz. siRNA (30 nmol/kg) or negative control siRNA (Santa Cruz) were injected via the tail vein every five days and starting three days before TAC, as previously demonstrated to be effective in heart (3, 4).

Efficacy of siRNA approach was evaluated by injecting an additional group of mice with a FITC-conjugated siRNA (Santa Cruz) and easily visualized in cardiac sections by green fluorescence.

Conventional and Strain Echocardiographic analysis

Conventional echocardiographic analysis was performed in mice anesthetized with tribromoethanol (175 mg/kg), using a Vevo 770 device equipped with a 30 MHz probe and a Vevo 2100 device equipped with 18-38MHz linear-array transducer with a digital ultrasound system (Visualsonics inc., Canada). A left ventricular M-mode tracing was obtained using the 2D parasternal short axis
imaging as a guide. End-diastolic interventricular septum (IVSd), posterior wall thicknesses (LVPWd), and left ventricular internal diameter (LVIDd) were measured. Relative wall thickness (RWT), ejection fraction (EF%) and fractional shortening (FS%) were calculated according to standard formulas.

Strain analysis was performed in mice anesthetized with tribromoethanol (175 mg/kg), using a Vevo 2100 device equipped with 18-38MHz linear-array transducer with a digital ultrasound system (Visualsonics inc., Canada). Using speckle-tracking– based strain analysis of 2D gray scale echocardiographic images acquired from the parasternal long- and short-axis views, strain and SR were quantified in the longitudinal and radial axes, as described by Bauer et al. (5). Accordingly with myocardial fiber orientation at varying levels of the LV wall, longitudinal strain is most representative of myocardial shortening at the level of the endocardium, whereas radial strain is more reflective of shortening at the level of the mesocardium. All images were acquired at a high frame rate (about 200 frames per second).

**Neonatal mouse cardiomyocytes**

Cardiomyocytes were obtained by hearts collected from 1 day old mice, through enzymatic digestion O.N. at 4°C in 0.5 mg/mL Trypsin (Sigma) HBSS, followed by 4 dissociation cycles with 240 U/mL Collagenase type II (Worthington) in HBSS. Cells attaching to tissue culture plates were discarded as non cardiomyocyte. Cardiomyocytes were plated on gelatin/fibronectin coated coverslip and maintained in DMEM-M199 medium, 10% Horse Serum (Gibco) and 5% FBS (Gibco). Cells were maintained for 48 h in serum free medium in presence of recombinant PlGF (R&D) to test its effects on cardiomyocytes growth. Then areas of cells positive to α-actinin (Sigma) staining were measured using MetaMorph Software.

**Immunohistochemistry, histology in situ hybridization and image analysis**

For immunohistochemical and histological analysis hearts were removed after diastolic arrest (KCl 1M) and then removed, post-fixed overnight in PBS 4% PFA, placed in 20% sucrose/0.1M phosphate buffer for 24 hours and cryosectioned (20 μm).
Myocyte cross-sectional area and microvascular density were examined on serial sections stained with Rhodamine-Wheat Germ Agglutinin (1:50; Vector) and Griffonia Simplicifolia Alexa488-lectin (1:50; Invitrogen) to visualize carbohydrate on plasma membrane and vessels, respectively. Sections were also stained with anti-CD68 (1:200; AbD Serotec), anti-TACE (1:100; Abcam), anti-α-actinin (1:200; Sigma), followed by the appropriate secondary. All sections were coverslipped using DAPI mounting media (Vector Laboratories). Fibrosis was assessed on sections stained with SiriusRed and cover-slipped with VectaShield Hard Set.

For in situ hybridization, hearts were post-fixed in PFA 4% and 10 cryosections were dried for 30’ in oven at 55°C. Before hybridization slides were digested with Proteinase K (20µg/ml; Sigma) and treated with 0.2% Glycin/PBS. Then sections were fixed with 4% PFA and 0.2% glutaraldehyde for 15’ and acetylated with 0.25% acetic anhydride in 0.1 M Tris-HCl. DIG labeled probes were incubated in hybridization mixture overnight at 70°C. The slides were then washed in 2X SSC followed by incubation at 65°C in 2X SSC containing 50% Formamide. Slides were rinsed in PBS-Tween and incubated in B-block for 1 hour, followed by a second incubation in anti-DIG alkaline phosphatase (1:500, Roche Biochemicals). After rinses, the slides were dipped in NTM (100 mM Tris, 100 mM NaCl, 50 mM MgCl2) and incubated overnight in NBT/BCIP (1:50, Roche Biochemicals) in NTM. The day after, slides were rinsed in PBS and dehydrated in graded ethanol, followed by rinses in xylene and mounted with Eukitt.

All images were captured using a DMI3000B Leica fluorescence/optical microscope provided of Leica cameras (Leica Microsystems, Wetzlar, Germany) and processed for quantitative analyses with the Leica Application Suite (LAS V3.3) Image Analysis.

**RNA, Reverse transcription, quantitative PCR and PCR array**

TRIzol reagent (Invitrogen, Eugene, OR) was used for RNA extraction and cDNA was obtained using Superscript III kit (Invitrogen, Eugene, OR), according to manufacturer’s instructions. Real-time PCR was performed with SYBR green PCR master mix, using an ABI Prism 7500 Sequence Detection System (Applied Biosystems Inc, Foster City, CA). Primer sequences were listed in
Supplementary Table 3. Samples were run in triplicate and gene expression levels were determined using the Relative Quantification (\(-\Delta\Delta Ct\)) Study of Applied Biosystems 7500 System SDS Software. For ECM and Adhesion Molecules analysis, Mouse PCR Array (PAMM-013, SABiosciences) was used according to manufacturer’s instructions.

**Western Blotting**

Heart proteins were extracted with lysis buffer (25mM Tris, 150mM NaCl, 1mM each of NaF, Na$_3$VO$_4$, EDTA and Sigma protease inhibitor cocktail) and centrifuged at 14,000xg for 30 min. Where indicated cytosolic and membrane fractions were obtained by ultracentrifugation.

Proteins were resolved by SDS-PAGE followed by Western blotting using the following antibodies: PIGF (1:200, sc-1882, Santa Cruz); CD68 (1:200, MCA1957ZX, AbD Serotec); C-term Flt-1 (1:250, sc-316, Santa Cruz); N-term Flt-1 (1:250, sc-31173, Santa Cruz); Notch-1 (1:200, sc-6015-R, Santa Cruz), TIMP-3 (1:200, sc-6836, Santa Cruz), Vinculin (1:5000). Secondary antibodies used were: donkey anti-rabbit (1:5000) and sheep anti-mouse (1:2500), from GE Healthcare; donkey anti-goat (1:10000) from SantaCruz. Protein detection was performed with ECL kit (Amersham) and densitometry was obtained with the NIH Image 1.61 software.

**Gelatin Zymography**

Hearts were homogenized in extraction buffer and centrifuged at 13000g. Proteins were separated under non denaturing conditions in Gelatin (3 mg/ml) containing gel. After electrophoresis, gels were washed for 30’ in a buffer containing Triton X-100, followed by incubation in Developing solution (Tris 50 mM, NaCl 200 mM, CaCl$_2$ 5 mM, BriJ 0.025) for 1 hour. Gels were then incubated overnight in the same buffer at 37°C. Gels were stained with Coomassie Blue 0.5% for 1 hour and destained by acetic acid in methanol and H$_2$O (200:40:160) for 1 hour, to visualize bands with gelatinolytic activity.

**TNF-alpha Assay and TACE Activity**
TNF-α levels were measured in triplicate for each cardiac tissue, extracted with a buffer containing PBS and protease inhibitor cocktail (Sigma), by a high sensitivity ELISA kit (Endogen, Pierce). Results are expressed as pg/mg of total proteins (measured by Bradford Assay kit; Biorad). TACE activity was measured in hearts lysates, processed with an alpha-secretase Activity Kit (Anaspec), following manufacturer’s instructions.
Echocardiographic analysis at different time points after Aortic Banding in PLGF KO and WT Mice.

<table>
<thead>
<tr>
<th></th>
<th>PLGF WT</th>
<th></th>
<th></th>
<th></th>
<th>PLGF KO</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>TAC 1wk</td>
<td>TAC 2wk</td>
<td>TAC 4wk</td>
<td>Basal</td>
<td>TAC 1wk</td>
<td>TAC 2wk</td>
<td>TAC 4wk</td>
</tr>
<tr>
<td>LVID (mm)</td>
<td>3.48 ± 0.12</td>
<td>3.24 ± 0.10</td>
<td>3.24 ± 0.20</td>
<td>3.35 ± 0.11</td>
<td>3.56 ± 0.06</td>
<td>3.79 ± 0.05 *</td>
<td>3.95 ± 0.09 *#</td>
<td>3.97 ± 0.09 *#</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.68 ± 0.02</td>
<td>1.05 ± 0.04</td>
<td>1.10 ± 0.09 *</td>
<td>1.09 ± 0.10 *</td>
<td>0.70 ± 0.01</td>
<td>0.88 ± 0.02*</td>
<td>0.87 ± 0.02 #</td>
<td>0.83 ± 0.02 #</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.72 ± 0.02</td>
<td>0.95 ± 0.04 *</td>
<td>1.02 ± 0.03 *</td>
<td>1.01 ± 0.09 *</td>
<td>0.73 ± 0.01</td>
<td>0.85 ± 0.01 *#</td>
<td>0.84 ± 0.01 *#</td>
<td>0.82 ± 0.03 *#</td>
</tr>
<tr>
<td>RWT</td>
<td>0.40 ± 0.01</td>
<td>0.62 ± 0.03 *</td>
<td>0.67 ± 0.07 *</td>
<td>0.63 ± 0.04 *</td>
<td>0.40 ± 0.01</td>
<td>0.46 ± 0.01 *#</td>
<td>0.44 ± 0.01 *#</td>
<td>0.42 ± 0.02 *#</td>
</tr>
<tr>
<td>LV % FS</td>
<td>48 ± 1</td>
<td>48 ± 2</td>
<td>48 ± 1</td>
<td>50 ± 2</td>
<td>48 ± 3</td>
<td>40 ± 1 *.#</td>
<td>38 ± 1 *.#</td>
<td>34 ± 1 *.#</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>526 ± 7</td>
<td>529 ± 6</td>
<td>532 ± 18</td>
<td>526 ± 8</td>
<td>539 ± 9</td>
<td>528 ± 7</td>
<td>533 ± 15</td>
<td>524 ± 13</td>
</tr>
<tr>
<td>Ao Grad</td>
<td>-</td>
<td>85 ± 4</td>
<td>89 ± 5</td>
<td>93 ± 7</td>
<td>-</td>
<td>85 ± 3</td>
<td>90 ± 6</td>
<td>88 ± 8</td>
</tr>
</tbody>
</table>

TAC indicates aortic banding; all parameters indicated are LV diastolic values. LVID, LV internal diameter; IVS, intraventricular septum; LVPW, LV posterior wall; RWT, relative wall thickness; LV %FS, fractional shortening; HR, heart rate; Ao Grad, trans-stenotic gradient. Values are mean ± SEM. *P<0.01 vs basal values; #P<0.01 vs TAC WT.
**Table S2.**

Echocardiographic analysis at 6 weeks after Aortic Banding in PlGF KO and WT Mice.

<table>
<thead>
<tr>
<th></th>
<th><em>PIGF WT</em></th>
<th><em>PIGF KO</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>TAC</td>
</tr>
<tr>
<td>LVID (mm)</td>
<td>3.77 ± 0.09</td>
<td>3.24 ± 0.10</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.74 ± 0.02</td>
<td>1.06 ± 0.02 *</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.74 ± 0.01</td>
<td>1.06 ± 0.02 *</td>
</tr>
<tr>
<td>RWT</td>
<td>0.39 ± 0.01</td>
<td>0.59 ± 0.02 *</td>
</tr>
<tr>
<td>LV % FS</td>
<td>44 ± 3</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Ao Grad</td>
<td>-</td>
<td>79 ± 3</td>
</tr>
</tbody>
</table>

TAC indicates aortic banding; all parameters indicated are LV diastolic values. LVID, LV internal diameter; IVS, intraventricular septum; LVPW, LV posterior wall; RWT, relative wall thickness; LV %FS, fractional shortening; HR, heart rate; Ao Grad, trans-stenotic gradient. Values are mean ± SEM. *P<0.01 vs basal values; #P<0.01 vs TAC WT.
Table S3.

Speckle-Tracking-Based Strain analysis at 6 weeks after Aortic Banding in PlGF KO and WT Mice.

<table>
<thead>
<tr>
<th></th>
<th>PlGF WT</th>
<th>PlGF KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>TAC</td>
</tr>
<tr>
<td><strong>Global</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Longitudinal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>-14.06 ± 2.55</td>
<td>-12.71 ± 1.17</td>
</tr>
<tr>
<td>SR</td>
<td>-4.95 ± 0.25</td>
<td>-4.88 ± 0.88</td>
</tr>
<tr>
<td>Velocity</td>
<td>0.84 ± 0.11</td>
<td>0.74 ± 0.07</td>
</tr>
<tr>
<td>Displacement</td>
<td>0.19 ± 0.03</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td><strong>Radial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>24.67 ± 3.67</td>
<td>19.66 ± 2.00</td>
</tr>
<tr>
<td>SR</td>
<td>5.57 ± 1.11</td>
<td>5.01 ± 0.53</td>
</tr>
<tr>
<td>Velocity</td>
<td>1.08 ± 0.09</td>
<td>1.06 ± 0.06</td>
</tr>
<tr>
<td>Displacement</td>
<td>0.42 ± 0.04</td>
<td>0.44 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *P<0.01 vs Sham of the same genotype; #P<0.01 vs TAC WT.
### Table S4.

**PCR array analysis for ECM and Adhesion molecules in 1-week TAC WT and PlGF KO Mice.**

<table>
<thead>
<tr>
<th>GENE NAME</th>
<th>PlGF WT TAC</th>
<th>PlGF KO TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adamts1</td>
<td>3.33</td>
<td>1.21</td>
</tr>
<tr>
<td>A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1</td>
<td>1.76</td>
<td>0.62</td>
</tr>
<tr>
<td>A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 2</td>
<td>0.83</td>
<td>0.71</td>
</tr>
<tr>
<td>A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 3</td>
<td>2.18</td>
<td>0.70</td>
</tr>
<tr>
<td>Cadherin 1</td>
<td>5.38</td>
<td>0.36</td>
</tr>
<tr>
<td>Cadherin 2</td>
<td>0.71</td>
<td>1.61</td>
</tr>
<tr>
<td>Cadherin 3</td>
<td>1.76</td>
<td>1.36</td>
</tr>
<tr>
<td>Cadherin 4</td>
<td>4.20</td>
<td>3.43</td>
</tr>
<tr>
<td>Collagen, type I, alpha 1</td>
<td>2.64</td>
<td>0.90</td>
</tr>
<tr>
<td>Collagen, type II, alpha 1</td>
<td>1.16</td>
<td>1.20</td>
</tr>
<tr>
<td>Collagen, type III, alpha 1</td>
<td>3.23</td>
<td>0.46</td>
</tr>
<tr>
<td>Collagen, type IV, alpha 1</td>
<td>1.08</td>
<td>0.66</td>
</tr>
<tr>
<td>Collagen, type IV, alpha 2</td>
<td>1.00</td>
<td>0.52</td>
</tr>
<tr>
<td>Collagen, type IV, alpha 3</td>
<td>1.54</td>
<td>1.31</td>
</tr>
<tr>
<td>Collagen, type V, alpha 1</td>
<td>2.05</td>
<td>1.23</td>
</tr>
<tr>
<td>Collagen, type VI, alpha 1</td>
<td>1.16</td>
<td>0.39</td>
</tr>
<tr>
<td>Fibronectin 1</td>
<td>4.45</td>
<td>1.14</td>
</tr>
<tr>
<td>Intercellular adhesion molecule 1</td>
<td>1.12</td>
<td>0.70</td>
</tr>
<tr>
<td>Integrin alpha 2</td>
<td>1.35</td>
<td>1.88</td>
</tr>
<tr>
<td>Integrin alpha 3</td>
<td>0.80</td>
<td>2.13</td>
</tr>
<tr>
<td>Integrin alpha 4</td>
<td>0.33</td>
<td>0.30</td>
</tr>
<tr>
<td>Integrin alpha 5 (fibronectin receptor alpha)</td>
<td>1.04</td>
<td>2.22</td>
</tr>
<tr>
<td>Integrin alpha E, epithelial-associated</td>
<td>1.87</td>
<td>1.64</td>
</tr>
<tr>
<td>Integrin alpha L</td>
<td>0.94</td>
<td>0.26</td>
</tr>
<tr>
<td>Integrin alpha M</td>
<td>1.72</td>
<td>0.94</td>
</tr>
<tr>
<td>Integrin alpha V</td>
<td>1.55</td>
<td>1.09</td>
</tr>
<tr>
<td>Integrin alpha X</td>
<td>1.02</td>
<td>0.65</td>
</tr>
<tr>
<td>Integrin beta 1 (fibronectin receptor beta)</td>
<td>1.08</td>
<td>1.27</td>
</tr>
<tr>
<td>Integrin beta 2</td>
<td>1.18</td>
<td>0.28</td>
</tr>
<tr>
<td>Integrin beta 3</td>
<td>1.69</td>
<td>1.39</td>
</tr>
<tr>
<td>Integrin beta 4</td>
<td>1.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Laminin, alpha 1</td>
<td>8.61</td>
<td>0.94</td>
</tr>
<tr>
<td>Laminin, alpha 2</td>
<td>0.88</td>
<td>0.58</td>
</tr>
<tr>
<td>Laminin, alpha 3</td>
<td>0.84</td>
<td>0.71</td>
</tr>
<tr>
<td>Laminin, beta 2</td>
<td>0.74</td>
<td>0.62</td>
</tr>
<tr>
<td>Laminin, beta 3</td>
<td>0.56</td>
<td>1.91</td>
</tr>
<tr>
<td>Laminin, gamma 1</td>
<td>1.17</td>
<td>2.38</td>
</tr>
<tr>
<td>Matrix metallopeptidase 10</td>
<td>4.26</td>
<td>0.90</td>
</tr>
<tr>
<td>Matrix metallopeptidase 11</td>
<td>0.52</td>
<td>0.32</td>
</tr>
<tr>
<td>Matrix metallopeptidase 12</td>
<td>3.83</td>
<td>0.46</td>
</tr>
<tr>
<td>Matrix metallopeptidase 13</td>
<td>0.58</td>
<td>0.62</td>
</tr>
<tr>
<td>Matrix metallopeptidase 14 (membrane-inserted)</td>
<td>1.61</td>
<td>1.97</td>
</tr>
<tr>
<td>Matrix metallopeptidase 2</td>
<td>0.78</td>
<td>0.61</td>
</tr>
<tr>
<td>Matrix metallopeptidase 3</td>
<td>1.10</td>
<td>2.13</td>
</tr>
<tr>
<td>Matrix metallopeptidase 7</td>
<td>4.60</td>
<td>1.24</td>
</tr>
<tr>
<td>Matrix metallopeptidase 8</td>
<td>2.93</td>
<td>0.12</td>
</tr>
<tr>
<td>Matrix metallopeptidase 9</td>
<td>0.54</td>
<td>0.40</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>0.99</td>
<td>0.36</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase 2</td>
<td>0.94</td>
<td>1.01</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase 3</td>
<td>0.85</td>
<td>3.71</td>
</tr>
</tbody>
</table>

Data are presented as fold changes respect sham animals of the same genotype of one representative experiment of n=3.
Figure 1S

A

Percent survival.

B

$\text{PGF WT}$ vs $\text{PGF KO}$

C

$\text{LVV (mg/dL)}$

D

$\text{LVV (mg/dL)}$

- Sham
- TAC 6 wk
Figure 2S

A. Western blot analysis of PI GF levels in WT and KO mice with or without TAC. p-PI GF levels were significantly higher in TAC mice compared to sham controls (p=0.024).

B. Quantification of cardiomyocyte area µm². TAC mice showed a significant increase in cardiomyocyte area compared to sham controls.

C. Maximum contrast intensity in PI GF WT and KO mice with or without TAC.

D. Immunoblot analysis of HIF-1α levels in WT and KO mice with or without TAC. TAC mice showed a significant increase in HIF-1α levels.

E. Representative images of hypoxprobe staining in WT and KO mice with or without TAC. TAC mice showed increased hypoxia staining.

F. Western blot analysis of VEGF-A and VEGF-B levels in WT and KO mice with or without TAC. VEGF-A and VEGF-B levels were significantly higher in TAC mice compared to sham controls.
Figure Legends

Figure 1S. A. Survival curve of KO (n=16) and WT (n=12) mice. *p<0.05 vs WT mice. B. Serum levels of Troponin I in KO and WT mice after 6 weeks of sham/TAC surgery. *p<0.01 vs sham PlGF KO and #p<0.01 vs TAC WT. C, D. Ratio of LV weight/body weight (C) and lungs weight/body weight (D) at the moment of sacrifice. *p<0.05 vs sham of the same genotype and #p<0.05 vs TAC WT.

Figure 2S. A. Representative western blot and ratio of phosphorylated Akt levels normalized to total Akt in 1 week TAC/sham KO and WT mice (n=3 for each group). B. Effect of increasing doses of recombinant PlGF on isolated mouse cardiomyocytes. *p<0.05 vs starv. C. Cardiac tissue perfusion measured by echocardiographic contrast agent in 1 week TAC/sham KO and WT mice (n=4 for each group). *p<0.01 vs sham and *p<0.05 vs TAC WT. D. Representative western blot of HIF-1α levels normalized for β-actin from cardiac lysates of animals exposed to TAC/sham surgery (n=3 for each group). E. Representative photomicrographs of cardiac sections from PlGF KO and WT subjected to 1 week of TAC/sham stained for HIF-1α (top panel; scale bar 50 μm) and Hypoxyprobe (bottom panel; scale bar 100 μm). F. Representative western blot and ratio of VEGF-A and VEGF-B levels normalized to vinculin in cardiac lysates of animals exposed to 1 week TAC/sham KO and WT mice (n=3 for each group).

Figure 3S. A. Representative western blot and ratio of Timp-3 levels normalized to vinculin in cardiac lysates of animals treated with Timp-3 siRNA and exposed to TAC for 1 day (lane 3) and 1 week (lane 4) as compared to TAC KO treated with neg Ctrl siRNA (lane 2) and TAC WT (lane 1) (n=3 for each group). *p<0.05 vs TAC WT, #p<0.05 vs neg Ctrl KO.
Supplemental References


