Published in final edited form as:

Biotechnol Bioeng. 2009 July 1; 103(4): 796–807. doi:10.1002/bit.22310.

Phthalimide neovascular factor 1 (PNF1) modulates MT1-MMP activity in human microvascular endothelial cells

Kristen A. Wiegand^{*},

Department of Biomedical Engineering, University of Virginia, Box 800759, Health System, Charlottesville, VA 22908, Phone: 434-982-1587, Fax: 434-982-3870

Erwin P. Gianchandani^{*},

Department of Biomedical Engineering, University of Virginia, Box 800759, Health System, Charlottesville, VA 22908, Phone: 434-982-6267, Fax: 434-982-3870

Rebekah A. Neal,

Department of Biomedical Engineering, University of Virginia, Box 800759, Health System, Charlottesville, VA 22908, Phone: 434-982-1587, Fax: 434-982-3870

Mikell A. Paige,

Lombardi Comprehensive Cancer Center, Drug Discovery Program, Department of Oncology, Georgetown University Medical Center, New Research Building, EP07, 3970 Reservoir Road, Washington, DC 20057, Phone: 202-687-8605

Milton L. Brown,

Lombardi Comprehensive Cancer Center, Drug Discovery Program, Department of Oncology, Georgetown University Medical Center, New Research Building, EP07, 3970 Reservoir Road, Washington, DC 20057, Phone: 202-687-8605

Jason A. Papin[†], and

Cardiovascular Research Center, Department of Biomedical Engineering, University of Virginia, Box 800759, Health System, Charlottesville, VA 22908, Phone: 434-924-8195, Fax: 434-982-3870

Edward A. Botchwey[†]

Department of Orthopaedic Surgery, Department of Biomedical Engineering, University of Virginia, Box 800759, Health System, Charlottesville, VA 22908, Phone: 434-243-9846, Fax: 434-982-3870

Abstract

We are creating synthetic pharmaceuticals with angiogenic activity and potential to promote vascular invasion. We previously demonstrated that one of these molecules, phthalimide neovascular factor 1 (PNF1), significantly expands microvascular networks *in vivo* following sustained release from poly(lactic-co-glycolic acid) (PLGA) films. In addition, to probe PNF1 mode-of-action, we recently applied a novel pathway-based compendium analysis to a multi-timepoint, controlled microarray dataset of PNF1-treated (versus control) human microvascular endothelial cells (HMVECs), and we identified induction of tumor necrosis factor-alpha (TNF- α) and, subsequently, transforming growth factor-beta (TGF- β) signaling networks by PNF1. Here we validate this microarray data-set with quantitative real-time polymerase chain reaction (RT-PCR) analysis. Subsequently, we probe this dataset and identify three specific TGF- β -induced genes with regulation by PNF1 conserved over multiple timepoints—amyloid beta (A4) precursor

[†]Corresponding authors: papin@virginia.edu; botchwey@virginia.edu.

^{*}These authors contributed equally to this work.

protein (APP), early growth response 1 (EGR-1), and matrix metalloproteinase 14 (MMP14 or MT1-MMP)—that are also implicated in angiogenesis. We further focus on MMP14 given its unique role in angiogenesis, and we validate MT1-MMP modulation by PNF1 with an *in vitro* fluorescence assay that demonstrates the direct effects that PNF1 exerts on functional metalloproteinase activity. We also utilize endothelial cord formation in collagen gels to show that PNF1-induced stimulation of endothelial cord network formation *in vitro* is in some way MT1-MMP-dependent. Ultimately, this new network analysis of our transcriptional footprint characterizing PNF1 activity 1–48 h post-supplementation in HMVECs coupled with corresponding validating experiments suggests a key set of a few specific targets that are involved in PNF1 mode-of-action and important for successful promotion of the neovascularization that we have observed by the drug *in vivo*.

Keywords

Network analysis; transcriptional profiling; angiogenesis; matrix metalloproteinase; small molecule; drug discovery

INTRODUCTION

Many significant age-related diseases arise from impaired or abnormal function of microvasculature, the complex network of small vessels (~100 μm) that transports blood to and from tissues. Therefore, effective strategies to promote vascularization are a critically important clinical need with potential to treat many significant disease pathologies, including heart disease, peripheral limb ischemia, osteonecrosis and diabetic wounds (Brey et al. 2005; Madeddu 2005). Despite the large number of patients suffering from these diseases and enormous research expenditures supporting basic science underlying their pathologies, available treatments to address vascular insufficiency have limited effectiveness. Additionally, mastery of angiogenic control will be essential for the future success of large-scale tissue-engineered constructs, as heralded by a recent review on strategic directions for the field of tissue engineering (Johnson et al. 2007).

The motivation for the current study stems from novel discoveries by our group of several small molecules that mimic the effects of growth factors commonly proposed for therapeutic neovascularization (Wieghaus et al. 2006; Wieghaus et al. 2005). Previous studies of drug optimization by various substitutions of a thalidomide (2-(2,6-dioxo-3-piperidyl)isoindole-1,3-dione) scaffold have led to the development of phthalimide neovascular factor 1 (PNF1, previously known as SC-3-149). Thalidomide is a potent anti-angiogenic agent that is hypothesized to function as an extracellular matrix-stabilizer (D'Amato et al. 1994; Gullestad et al. 2005). We have shown that appropriate engineered structural analogues of thalidomide, such as PNF1, enhance microvascular endothelial survival and growth, promote wound healing behavior and formation of capillary-like networks *in vitro*, and increase microvessel density *in vivo* following bolus injection into rat mesentery with limited systemic effects (Wieghaus et al. 2006; Wieghaus et al. 2005). These new drugs are more easily synthesized relative to polypeptide growth factors commonly proposed for therapeutic induction of angiogenesis, and they also accommodate a wider range of storage and processing methods for *in vivo* delivery.

We recently investigated the *in vivo* efficacy of PNF1 by releasing the drug from degradable poly(lactic-co-glycolic acid) (PLGA) polymer films in a murine dorsal skin-fold window chamber model (see (Nickerson et al. 2008; Rucker et al. 2006)) and monitoring PNF1-stimulated tissues for angiogenic and arteriogenic effects during early-stage (seven-day) remodeling (Wieghaus et al. 2008b). We identified six-fold increases in metrics of

angiogenesis and arteriogenesis, thus validating the ability of PNF1 to induce angiogenesis *in vivo* (Wieghaus et al. 2008b). In addition, as proper utilization of PNF1 as a vascular therapy requires characterization of the underlying mechanism by which the drug induces angiogenesis, we performed transcriptional profiling using oligonucleotide microarrays and implemented a novel pathway-based compendium analysis (Wieghaus et al. 2008a). Specifically, we overlaid microarray data of PNF1-stimulated and control human microvascular endothelial cells (HMVECs) on literature-derived angiogenic pathways, and we ultimately concluded that tumor necrosis factor- α (TNF- α) pathway components are most affected by PNF1 stimulation at early timepoints followed by transforming growth factor (TGF- β) pathway components at later timepoints (Wieghaus et al. 2008a). This analysis extended earlier work in which we compared PNF1-stimulated with control HMVECs at a single timepoint (24 h) and identified TGF- β pathway involvement in PNF1 function (Wieghaus et al. 2007).

Here, we attempt to more precisely characterize the mechanism of PNF1 across a wider range of signaling and transcriptional regulatory activity. Specifically, we applied network analysis to the comparative microarray data of HMVECs following stimulation with PNF1 (versus control) for 1, 2, 4, 8, 16, 24, and 48 h (see (Wieghaus et al.) and (Wieghaus et al. 2008a) for the original dataset). Of all the gene transcripts interrogated as part of a standardized human genome microarray (approximately 47,000 such transcripts), we identified three with differential regulation at a maximum of five of the seven timepoints that we studied. These included amyloid beta (A4) precursor protein (APP), early growth response 1 (EGR-1), and matrix metalloproteinase 14 (MMP14 or MT1-MMP), all of which are implicated in angiogenesis. As functional metalloproteinase (MMP) activity has been clearly implicated in the angiogenic response, in that MMP-deficient mice have exhibited delayed or diminished angiogenesis (see (Lehr et al. 1993) and (Ichioka et al. 2002)), we further validated the effect of PNF1 on MT1-MMP activity through independent *in vitro* assays.

Ultimately, taken together, our work demonstrates that PNF1, which induces angiogenesis *in vivo* and has been hypothesized to function by first affecting the TNF- α pathway which in turn induces the TGF- β pathway, modulates MT1-MMP activity *in vitro*. In particular, we have newly identified MT1-MMP as a possible PNF1 target or mediator.

METHODS

Cell culture and RNA isolation

Human microvascular endothelial cells (HMVECs) (Cambrex, Walkersville, MD) were cultured using the procedure previously described (Wieghaus et al. 2007; Wieghaus et al. 2008a). Total RNA from the cultures was isolated 1, 2, 4, 8, 16, 24, and 48 h post-supplementation using an RNeasy kit (Qiagen, Inc., Valencia, CA) (Wieghaus et al. 2007; Wieghaus et al. 2008a).

Gene array and analysis

At each timepoint, RNA samples were hybridized to Affymetrix Human Genome U133 Plus 2.0 probe arrays as previously described (Wieghaus et al. 2007; Wieghaus et al. 2008a). The arrays were washed, stained, and scanned, and acceptable image files were analyzed to generate raw data in the form of “CEL” files. Quality control was performed using the Microarray Analysis Suite 5.0 (MAS 5.0; Affymetrix, Santa Clara, CA), as previously explained (Wieghaus et al. 2007; Wieghaus et al. 2008a). Expression profiles were generated and analyzed using the procedure previously described (Wieghaus et al. 2007; Wieghaus et al. 2008a). Briefly, using MAS 5.0, each gene transcript was detected as

“present,” “absent,” or “marginal,” and the change between the experimental (PNF1-stimulated) and baseline (control) conditions for each gene transcript at each timepoint was assigned to one of seven standard categories, i.e., increase, marginal increase, no change, marginal decrease, and decrease, depending on the *p*-value (calculated using the Wilcoxon signed rank test implemented by the GeneChip Operating Software (GCOS; Affymetrix)). The ranges for each call were 0.0000 to 0.0025, 0.0025 to 0.0030, 0.0030 to 0.9970, 0.9970 to 0.9975, and 0.9975 to 1.0000, respectively. These data were then inputted into dChip version 1.3 (Li and Wong 2001), where they were normalized using the invariant set approach. The normalized expression data were used for the network analysis described below. These expression data will be deposited into the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>).

Quantitative real-time polymerase chain reaction (RT-PCR) as validation of the gene arrays

cDNA (from 0.01 µg/µL RNA solution at 1, 2, 4, 8, 16, and 48 h) was synthesized with a Bio-Rad iScript kit (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions. Five microliters of the subsequent cDNA template was added to a reaction well containing 20 µL of 100 nM forward and reverse gene-specific primers, 4 mM MgCl₂ and SYBR Green Supermix (Bio-Rad), including SYBR green, dNTPs, Taq enzyme and reaction buffer. PCR reactions were carried out using an iCycler thermal cycler (Bio-Rad): an initial denaturing step was performed at 95°C, prior to 40 cycles of 95°C for 10 sec, *x*°C for 30 sec, and 72°C for 45 sec, where *x* is the specific annealing temperature of the primer set (see Table 1).

Identification of genes regulated coordinately across multiple timepoints

The gene transcripts identified by microarray analysis as differentially (up or down) regulated at each timepoint (1, 2, 4, 8, 16, and 48 h) were evaluated with further network and gene ontology analyses as previously described for the published 24 h timepoint (Wieghaus et al. 2007). For these analyses, the Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com), including the Ingenuity Pathways Knowledge Base (IPKB), was employed. Graphical representations of the molecular relationships between gene transcripts were generated by IPA to further investigate the significantly regulated networks. (See (Wieghaus et al. 2007) and (Wieghaus et al. 2008a) for further details about IPA and the IPKB.)

Once top-scoring networks and functions were identified at each timepoint, these results were compared to investigate key processes regulated by treatment with the drug temporally. The most regulated genes, most connected genes, and most affected molecular and cellular functions at each timepoint were determined; and significantly differentially regulated genes across multiple timepoints were identified.

Basic network and function characteristics at each timepoint

In considering the most connected genes at each timepoint, a cutoff of four connections was chosen after the data were evaluated. Additionally, to indicate the significance of the number of connections for a given gene at a given timepoint, this number was normalized to the total number of connections for the gene in the IPKB. Ultimately, we highlight here the 10 most connected genes at each timepoint (see Appendix for a more complete listing) as well as the 10 most-regulated genes at each timepoint.

Genes differentially regulated at multiple timepoints

Gene transcripts that were differentially regulated (between PNF1-treated and negative control samples) at multiple timepoints were evaluated. Specifically, those transcripts that

were differentially regulated at any two, three, four, or five timepoints were identified. (No genes were differentially regulated at any six or all seven timepoints.) The genes that were differentially regulated at the maximum number of timepoints (five), including MMP14, were further investigated for their links to angiogenesis and involvement in drug mechanism.

MMP fluorogenic peptide substrate assay

The fluorescence-quenched MMP substrate Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (FS-6; R&D Systems, Minneapolis, MN) has been used to quantify MMP activity, including MT1-MMP activity, in cell cultures (Neumann et al. 2004). Confluent cultures of HMVECs in 96-well plates were supplemented with 100 μ L cell media containing vehicle control or 30 μ M PNF1. Human recombinant tissue inhibitor of metalloproteinase-1 or 2 (TIMP-1 or TIMP-2) (Sigma, Saint Louis, MO) was added at a final media concentration of 2.5 μ g/mL (Sodek et al. 2007). Substrate FS-6 was added at a final concentration of 5 μ M, and after 2 h incubation at 37°C with 5% CO₂, reactions were stopped with 3% (w/v) sodium acetate. Fluorescence was detected using a FLUOstar OPTIMA microplate reader (BMG Labtech, Durham, NC) with wavelengths of 320 nm (excitation) and 400 nm (emission). Data are reported as mean ($n = 6$) $\pm$ standard error, and the mean value was subtracted by background fluorescence for each sample.

Microwell collagen gel assay

Experiments in type I collagen were performed according to a protocol modified from (Bayless and Davis 2003). Type I rat tail collagen (BD Biosciences, Bedford, MA) gels (5 μ L at 2.5 mg/mL) were polymerized in 96-well plates (Nalge Nunc, Rochester, NY) at 37°C with 5% CO₂ for 45 min. Subsequently, 100 μ L volumes of HMVECs (15,000 cells/well) were added with 30 μ M PNF1 or vehicle control, as well as TIMP-1 or TIMP-2 and cultured for 24 h. Using a 10x objective on an Axioskop 40 microscope (Carl Zeiss) photographs of the gels were captured with a digital camera (Olympus), which were subsequently analyzed for number of cell-cell associations per well. Data are reported using mean ($n = 6$) $\pm$ standard error.

RESULTS

Comparative microarray analysis

To further elucidate the mechanism by which PNF1 functions, comparative microarrays were performed on control- and drug-treated cultures after 1, 2, 4, 8, 16, 24, and 48 h of stimulation (Wieghaus et al. 2007; Wieghaus et al. 2008a). Differential expression between control- and PNF1-stimulated culture samples was found in varying numbers of gene transcripts, characterized as increased or decreased according to p -value (see Figure 1a). The most gene transcripts with significantly differential expression levels occurred at 2 and 48 h (1422 and 1622 genes, respectively) (see Appendix for additional information about the 2 and 48 h Ingenuity Pathway Analysis (IPA)-derived networks).

Microarray validation with quantitative RT-PCR

Validation of 24 h microarray results was performed previously by quantitative RT-PCR analysis (see (Wieghaus et al. 2007)). To validate results obtained from the microarray experiments at the other time-points included in our study, we extended RT-PCR analysis to measure expression changes in TGF- β 1, TGF- β 1 receptor CD36, fibronectin 1 (FN1), and Kruppel-like factor 2 (KLF2) at 1, 2, 4, 8, 16, and 48 h post-supplementation. These genes were selected because of their known effects in angiogenic signaling pathways within endothelial cells. In general, RT-PCR demonstrated consistency with the microarray

experiments (see Table 2). In 18 of 22 (82%) assays performed, a gene product that was upregulated in the microarray experiment was also upregulated when using RT-PCR, and vice-versa. Furthermore, as we will describe below, our systems-based approach of overlaying differential gene expression on an interaction network accounts for global changes in transcription, thereby minimizing sensitivity to individual values that may be affected by experimental artifacts.

Network and gene ontology analyses

Coordinated expression levels of gene products regulated by PNF1 at each timepoint were examined using IPA, which uses molecular interactions described in literature to elucidate functional relationships among transcripts (see (Wieghaus et al. 2007) for more details pertaining to the 24 h timepoint). Networks with significant expression level changes were assigned a score greater than 3 ($1/10^3$ chance the networks and underlying fold changes were generated by chance); top-scoring network(s) at each timepoint were selected for further analysis (see Figure 1b).

Cellular processes associated with PNF1 stimulation were also identified at each timepoint (see Table 3). Each molecular/cellular function in the IPKB is given a significance *p*-value, calculated using the right-tailed Fisher's exact test (a measure of the likelihood that genes from the dataset participate in the corresponding function). The most significantly activated processes 1–48 h are critical to endothelial cells for angiogenesis, namely, cell death, cell-to-cell signaling and interaction, cellular assembly and organization, and cellular morphology. Although many different functional classifications are possible (the IPKB contains over 50 categories), the dataset inputted into IPA yielded a set of ontologies commonly associated with angiogenesis.

Single gene transcripts with the highest fold changes were isolated at each time-point (see Table 3). Importantly, most differentially-expressed transcripts have direct implications on the process of angiogenesis (see detailed listing in Appendix). After 1 h, both FN1 and syndecan-2 (SDC2) were highly-upregulated with fold changes of 22- and 19-fold, respectively. Both are integral to initial phases of angiogenesis: FN1 promotes elongation of developing microvessels via migratory endothelial recruitment while SDC2 is required for induction of angiogenesis (Essner et al. 2006;Nicosia et al. 1993). Conversely, after 48 h, genes with high expression fold changes were associated with later phases of angiogenesis. For example, collagen type III, alpha 1 (COL3A1), an important component of the arterial wall (see (Timur et al. 2005)), was upregulated 39-fold.

The most interconnected gene transcripts were also investigated at each timepoint (see Table 3, as well as images of top-scoring networks in Appendix), as they have the most signaling interactions with other expressed transcripts. Most are implicated in the angiogenic process as well. After 1 h, APP and jun oncogene (JUN) had the most interconnections. JUN (also known as AP-1) is a transcription factor that upregulates vascular endothelial growth factor (VEGF) during angiogenic processes (see (Josko and Mazurek 2004)), while APP stimulates endothelial proliferation, chemotaxis, and morphogenesis, as well as the formation of capillary-like structures (see (Boscolo et al. 2007)). After 48 h, gene products with the most interconnections were mitogen-activated protein kinase 8 (MAPK8, also known as JNK; 35 connections), epidermal growth factor receptor (EGFR; 27 connections), and VEGF (27 connections). MAPK8 is a signaling molecule of the TGF- β canonical pathway, and VEGF is a well-studied inducer of angiogenesis. EGFR induces endothelial proliferation, angiogenesis, cell mobility and inhibition of apoptosis (Ono and Kuwano 2006;Penault-Llorca et al. 2005).

Identification of genes with expression conserved over multiple timepoints

The numbers of gene transcripts that were differentially expressed at multiple timepoints, as assessed by a Wilcoxon signed rank test implemented by the GeneChip Operating Software (GCOS; Affymetrix), were evaluated. Transcripts were characterized as having differential expression changes at one, two, three, four, or five timepoints (see comprehensive graphs in Appendix); none of the transcripts was differentially expressed at any six or all seven timepoints. Only three gene products had conserved differential expression across five timepoints: APP, EGR-1, and MT1-MMP/MMP14 (see expression values graphed in Figure 2a). Each transcript has significant effects on the angiogenic process (see Figure 2b). As discussed above, APP is implicated in endothelial proliferation and chemotaxis (Boscolo et al. 2007). EGR-1 is a transcription factor activated in response to multiple extracellular agonists (growth factors and cytokines) (see (Fahmy et al. 2003)) after which it increases tissue perfusion in ischemic hindlimb (see (Lee et al. 2005)), stimulates angiogenesis (see (Bryant et al. 2000)), and controls expression of various angiogenic genes, including members of the TGF- β , PDGF, and bFGF pathways, MMPs, VEGF receptor Flt-1 (see (Fahmy et al. 2003)), and others (see (Lee et al. 2005)). Functionally, MT1-MMP is essential for extracellular matrix degradation to allow for endothelial invasion (Haas and Madri 1999; Heissig et al. 2003; Pepper 2001). Importantly, APP, EGR-1, and MT1-MMP are induced by TGF- β (see (Cheng and Lovett 2003; Chipuk et al. 2002; Harris-White et al. 1998)), which demonstrates the complementary nature of this experiment with our other findings.

Fluorogenic assay measuring MMP activity *in vitro*

To validate the effect of PNF1 on MT1-MMP activity, we used a fluorogenic assay measuring MMP activity *in vitro*. Fluorescence-quenched peptide substrates have been widely used to quantify the actual enzymatic activity of MMPs. The FS-6 substrate, in particular, has higher specificity for MT1-MMP than other fluorogenic substrates (Neumann et al. 2004). The peptide substrate contains a highly fluorescent 7-methoxycoumarin group that is efficiently quenched by resonance energy transfer to the 2,4-dinitrophenyl group, and can be used to measure peptidase activities (such as that of MMPs) that cleave an amide bond between the fluorescent group and the quencher groups, causing an increase in fluorescence. Samples supplemented with vehicle control for served to elucidate background MMP activity in HMVECs cultures. The quantified fluorescence observations were consistent with microarray data at three of four timepoints investigated with the assay (see Figure 3). Specifically, at 1 h and 4 h post-supplementation, relative FS-6 activity in PNF1-treated cultures was increased as compared with control ($*p < 0.05$), which is consistent with microarray-based findings. Likewise, after 2 h, PNF1-treated samples demonstrated 81% of the FS-6 fluorescence observed in control samples ($*p < 0.05$), which is also consistent with microarray experiments that suggested MT1-MMP mRNA was down-regulated by approximately -1.75-fold (or 0.57-fold) in PNF1-treated cultures after 2 h. Only at 8 h did the results of the fluorogenic assay differ from the microarray data, but there was no statistical significance in this set of fluorogenic data ($n = 6$).

Collagen gel assay

The relationship between PNF1 stimulation of HMVECs and MMP14 regulation was also validated with type I collagen gel invasion assays over 24 h. Formed from the main extracellular matrix (ECM) component, these gels allowed for three-dimensional endothelial culture and supported cell-cell associations between endothelial cells. MT1-MMP, the protein formed from the MMP14 transcript, is critical for endothelial invasion via ECM degradation (Chun et al. 2004); recent studies in Matrigel have demonstrated that MT1-MMP degrades the matrix and allows for formation of multi-cellular structures (Hotary et al. 2000). In this experiment, increases in endothelial invasion, or establishment of cell-cell

associations, are dependent upon MT1-MMP regulation and degradation of ECM. Many MMPs are soluble; however, MT1-MMP is membrane-bound. TIMP-1 is an endogenous inhibitor of many soluble MMPs, but not MT1-MMP. In contrast, TIMP-2 is an inhibitor of both soluble MMPs and membrane-bound ones like MT1-MMP. Therefore, while there is no MT1-MMP-specific inhibitor, contrasting results from the addition of either TIMP-1 or TIMP-2 to cell cultures allows for elucidation of MT1-MMP signaling activity. Figure 4a depicts the significant rounding of cells and lack of cell-cell associations seen in cultures treated with both PNF1 and TIMP-2, suggesting that MT1-MMP is critical for PNF1-stimulated endothelial invasion through type I collagen. Figure 4a also demonstrates the increase in the numbers of elongated HMVECs and cell-cell associations observed when cultures were stimulated with 30 μ M PNF1, as compared with vehicle control. This trend parallels that of previous experiments performed in Matrigel (Wieghaus et al. 2006). Cell invasion stimulated by PNF1 through collagen was not significantly inhibited by 2.5 μ g/mL TIMP-1 (Sodek et al. 2007). In contrast, the number of cell-cell associations significantly decreased ($p < 0.05$) with the addition of TIMP-2 at the same concentration (see Figure 4b). The number of cell-cell associations decreased when TIMP-1 or TIMP-2 was added to control cells as well (see Figure 4b), because basal MMP activity (not modulated by PNF1) was reduced by the proteases. However, importantly, the decrease was much more dramatic, and indeed statistically significant, in the case of PNF1-treated cells.

DISCUSSION

Tissue growth and regeneration in the adult requires a vasculature that meets the metabolic needs of the repairing tissue. Naturally-occurring vascular remodeling is often insufficient for management of ischemic diseases, as well as for tissue engineering and regenerative medicine applications where significant nutritive and metabolic demands are created (Berglund and Galis 2003; Bernas 2003; Freedman and Isner 2002; Hockel et al. 1993; Hughes et al. 2004). Therapeutic strategies that capitalize on the natural processes of angiogenesis and/or arteriogenesis are attractive, as they address treatment of vascular insufficiencies by enhancing blood flow to tissues (Freedman and Isner 2002; Takeshita et al. 1994).

We have previously demonstrated that PNF1 promotes endothelial proliferation, migration, and other cellular processes relevant to angiogenesis *in vitro* (see (Wieghaus et al. 2006)). We have also recently shown that PNF1 significantly expands microvascular networks *in vivo* by increasing microvessel length density and luminal diameter of local arterioles and venules (Wieghaus et al. 2008b). In addition, comparative transcriptional profiling of PNF1-stimulated (versus control) HMVECs coupled with a novel pathway-based compendium analysis identified induction of TNF- α and, subsequently, TGF- β pathways as integral to this PNF1 function (Wieghaus et al. 2008a). Here we further interrogated PNF1 mode-of-action and specifically identified drug-based modulation of MT1-MMP, a key component in the TGF- β pathway implicated in angiogenesis.

To identify key pathway components associated with PNF1 function, here we focused our network analysis on genes critical to their canonical pathways, allowing for isolation of a definitive number of TGF- β -related transcripts for future probing of PNF1 mechanism. We also elucidated gene products with differential expression conserved across a maximum number of timepoints, limiting focus to three gene products out of over 47,000 transcripts: APP, MT1-MMP (MMP14), and EGR-1. Notably, our network-based approach allowed us to eventually concentrate on three genes that were *not highly* regulated at any particular timepoint, that would not have been selected by simply picking genes at the top of a list rank-ordered on the basis of microarray fold change, and that demonstrate complex relationships among themselves. For example, EGR-1 binding to the MT1-MMP promoter is

critical for MT1-MMP signaling and matrix invasion (Haas 2005). Figure 2a shows that the expression levels of these three gene products are very similar across the investigated time frame. Each PNF1-regulated gene has substantial implications on angiogenesis, and hence, these genes may be critical for the actions of PNF1. As these gene products were consistently regulated by persistent stimulation with PNF1, these transcripts and their biological functions may offer significant insight into drug mechanism. While PNF1 increases MT1-MMP activity after 1, 4, and 8 hours, it is interesting to note that the novel angiogenic factor actually decreases MT1-MMP activity after 2 hours; these results are consistent with previous mRNA microarray experiments. Although MT1-MMP is critical for initiation of the angiogenic process via degradation of the ECM, the activity of the protein may be inhibited at various steps in the angiogenic cascade, as endothelial cells complete the process of invasion (van Hinsbergh and Koolwijk 2008). For example, it has been suggested that cells must mask MT1-MMP activity in cellular areas between the protruding cellular tip and posterior cellular area, where proteolytic molecules must neutralize existing cell-cell interactions before the cell can advance through the ECM (Wolf et al. 2007). Additionally, various mechanisms for temporary inactivation of MT1-MMP inactivation exist, including proteolytic processing (Golubkov et al. 2007), temporary binding of inhibitors, delay of dimerization (Itoh et al. 2006), and internalization (Itoh and Seiki 2006). While the signaling mechanisms driving MT1-MMP activity in human endothelial cells may not be completely understood, it is clear that gene expression levels are not the only factors influencing MT1-MMP in the endothelial milieu.

Furthermore, functional MMP activity has been clearly implicated in the angiogenic response, in that MMP-deficient mice exhibit delayed or diminished angiogenesis (Itoh et al. 1998; Stetler-Stevenson 1999; Vu et al. 1998). The initiating stimulus for vascular remodeling results in the formation of a migrating column of cells known as the vascular sprout; the advancing front of this cell column engages in proteolytic activity in order to traverse the extracellular matrix (Stetler-Stevenson 1999). Moreover, MT1-MMP (the protein product of the MMP14 transcript) activation of pro-MMP-2 in particular is critical for endothelial invasion in type I collagen, and MT1-MMP is the only matrix metalloproteinase with sufficient fibrinolytic activity for matrix invasion (Haas et al. 1998; Hiraoka et al. 1998). This model is dependent on TIMP-2, which at high concentrations is a natural inhibitor of MMP signaling. Binding of pro-MMP-2 to the MT1-MMP/TIMP-2 complex localizes the molecule to the cell surface, and a second, non-TIMP-2-bound MT1-MMP molecule cleaves it into its active form (Stetler-Stevenson 1999; Will et al. 1996). Treatment of endothelial cells with exogenous pro-MMP-2 induces dose-dependent morphologic changes, including tubule formation. When cultured in three-dimensional collagen I matrix, as opposed to on top of two-dimensional collagen-coated systems, pro-MMP-2 transcript levels increase, which is coordinate with similar modulation of MT1-MMP (Haas et al. 1998). Recently, it has been suggested that the regulation of MT1-MMP during therapeutic and pathologic vascular remodeling is more complex, and that, as described previously, the sign of gene regulation (e.g., up- vs. downregulation) critical for any particular vascular process is perhaps more ambiguous than previously thought (Overall and Kleifeld 2006).

Thus, to further validate the effect of PNF1 on MMP14/MT1-MMP gene regulation and protein activity as part of the drug's pro-angiogenic effects, we conducted two *in vitro* assays, namely a fluorogenic substrate experiment measuring relative levels of MMP activity and a three-dimensional culture assay in type I collagen. Results of the former demonstrated PNF1 modulation of MT1-MMP over the course of 1–8 h (four timepoints), which generally corresponded with relative levels of gene expression as measured by our microarray studies. These data were also validated by a collagen gel assay that showed that induction of cell-cell associations by PNF1 in the three-dimensional matrix was significantly

reduced by the addition of TIMP-1, which is known to inhibit MT1-MMP/MMP14; conversely, as a control, this effect was not observed with TIMP-2, which inhibits most MMPs but not MT1-MMP. These results parallel those of previous experiments (see (Wieghaus et al. 2006)) demonstrating statistically significant increases in cell-cell associations in Matrigel cultures after stimulation with PNF1, and also serve to validate the effect of PNF1 on MT1-MMP activity.

From these data, we can now begin to hypothesize a mechanism by which PNF1 may exert its pro-angiogenic effects on HMVECs. Highlights of the angiogenic process, including ECM degradation and endothelial proliferation and chemotaxis, are diagrammed in Figure 5. The points at which APP, EGR-1, and MT1-MMP/MMP14 have been documented to modulate vascular remodeling are overlayed on the diagram, illustrating how these three proteins collectively regulate processes involved in angiogenesis. Therefore, the previously documented pro-angiogenic effects of PNF1 *in vitro* (see (Wieghaus et al. 2006)) and *in vivo* (see (Wieghaus et al. 2008b)) may be elucidated by the coordinated regulation of APP, EGR-1, and MT1-MMP gene products at multiple timepoints over the course of 48 h after drug stimulation.

Comparing RNA expression at multiple timepoints spanning 1–48 h allowed several relevant timescales for angiogenesis to be studied. As a result, this expansion to a temporal profile of drug-stimulated transcription, coupled with our network analysis, suggested novel components of PNF1 mechanism that were not elucidated in previous experiments at a single timepoint (24 h only). The transcripts isolated by our temporal profile of PNF1 stimulation, especially MT1-MMP, may provide data influential to future therapeutic designs and strategies for drug dosage and release. Additionally, the elucidation of MT1-MMP regulation by PNF1 may offer new paradigms other than vascular remodeling for which the drug might be efficacious. For example, MT1-MMP has been demonstrated as a facilitator of leukocyte transmigration (see (Sithu et al. 2007)) and stromal cell trafficking (see (Neth et al. 2007)); hence, in future experiments, we may determine the potential of PNF1 as an effector of these important biological processes. Finally, independent of our PNF1 mode-of-action investigation, since the microarray data-set is the transcriptional footprint of a pro-angiogenic factor, it may also facilitate future interrogation of key angiogenic mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources for support of these studies include the National Institute of Arthritis and Musculoskeletal Disease (NIAMS) grant K01AR052352-01A1 to Dr. Botchwey. Kristen Wieghaus was also supported by the US Department of Education's Graduate Assistance in Areas of National Need (GAANN) funding. Erwin Gianchandani was supported by the National Institutes of Health (NIH) (GM08715/NIH Biotechnology Training Grant). We also thank Elizabeth Logsdon, Nicole Hastings, and Ryan Feaver for assisting with the RT-PCR experiments.

References

- Anderson CR, Hastings NE, Blackman BR, Price RJ. Capillary sprout endothelial cells exhibit a CD36 low phenotype: regulation by shear stress and vascular endothelial growth factor-induced mechanism for attenuating anti-proliferative thrombospondin-1 signaling. *Am J Pathol.* 2008; 173(4):1220–8. [PubMed: 18772338]
- Bayless KJ, Davis GE. Sphingosine-1-phosphate markedly induces matrix metallo-proteinase and integrin-dependent human endothelial cell invasion and lumen formation in three-dimensional

- collagen and fibrin matrices. *Biochem Biophys Res Commun.* 2003; 312(4):903–13. [PubMed: 14651957]
- Berglund JD, Galis ZS. Designer blood vessels and therapeutic revascularization. *Br J Pharmacol.* 2003; 140(4):627–36. [PubMed: 14534146]
- Bernas GC. Angiotherapeutics from natural products: from bench to clinics? *Clin Hemorheol Microcirc.* 2003; 29(3–4):199–203. [PubMed: 14724342]
- Boscolo E, Folini M, Nico B, Grandi C, Mangieri D, Longo V, Scienza R, Zampieri P, Conconi MT, Parnigotto PP, et al. Beta amyloid angiogenic activity in vitro and in vivo. *Int J Mol Med.* 2007; 19(4):581–7. [PubMed: 17334633]
- Brey EM, Uriel S, Greisler HP, McIntire LV. Therapeutic neovascularization: contributions from bioengineering. *Tissue Eng.* 2005; 11(3–4):567–84. [PubMed: 15869435]
- Bryant M, Drew GM, Houston P, Hissey P, Campbell CJ, Braddock M. Tissue repair with a therapeutic transcription factor. *Hum Gene Ther.* 2000; 11(15):2143–58. [PubMed: 11044915]
- Cheng S, Lovett DH. Gelatinase A (MMP-2) is necessary and sufficient for renal tubular cell epithelial-mesenchymal transformation. *Am J Pathol.* 2003; 162(6):1937–49. [PubMed: 12759250]
- Chipuk JE, Cornelius SC, Pultz NJ, Jorgensen JS, Bonham MJ, Kim SJ, Danielpour D. The androgen receptor represses transforming growth factor-beta signaling through interaction with Smad3. *J Biol Chem.* 2002; 277(2):1240–8. [PubMed: 11707452]
- Chun TH, Sabeh F, Ota I, Murphy H, McDonagh KT, Holmbeck K, Birkedal-Hansen H, Allen ED, Weiss SJ. MT1-MMP-dependent neovessel formation within the confines of the three-dimensional extracellular matrix. *J Cell Biol.* 2004; 167(4):757–67. [PubMed: 15545316]
- D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci U S A.* 1994; 91(9):4082–5. [PubMed: 7513432]
- Essner JJ, Chen E, Ekker SC. Syndecan-2. *Int J Biochem Cell Biol.* 2006; 38(2):152–6. [PubMed: 16198615]
- Fahmy RG, Dass CR, Sun LQ, Chesterman CN, Khachigian LM. Transcription factor Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumor growth. *Nat Med.* 2003; 9(8):1026–32. [PubMed: 12872165]
- Freedman SB, Isner JM. Therapeutic angiogenesis for coronary artery disease. *Ann Intern Med.* 2002; 136(1):54–71. [PubMed: 11777364]
- Golubkov VS, Chekanov AV, Shiryaev SA, Aleshin AE, Ratnikov BI, Gawlik K, Radichev I, Motamedchaboki K, Smith JW, Strongin AY. Proteolysis of the membrane type-1 matrix metalloproteinase prodomain: implications for a two-step proteolytic processing and activation. *J Biol Chem.* 2007; 282(50):36283–91. [PubMed: 17938169]
- Gullestad L, Ueland T, Fjeld JG, Holt E, Gundersen T, Breivik K, Folling M, Hodt A, Skardal R, Kjekshus J, et al. Effect of thalidomide on cardiac remodeling in chronic heart failure: results of a double-blind, placebo-controlled study. *Circulation.* 2005; 112(22):3408–14. [PubMed: 16301340]
- Haas TL. Endothelial cell regulation of matrix metalloproteinases. *Can J Physiol Pharmacol.* 2005; 83(1):1–7. [PubMed: 15759044]
- Haas TL, Davis SJ, Madri JA. Three-dimensional type I collagen lattices induce coordinate expression of matrix metalloproteinases MT1-MMP and MMP-2 in microvascular endothelial cells. *J Biol Chem.* 1998; 273(6):3604–10. [PubMed: 9452488]
- Haas TL, Madri JA. Extracellular matrix-driven matrix metalloproteinase production in endothelial cells: implications for angiogenesis. *Trends Cardiovasc Med.* 1999; 9(3–4):70–7. [PubMed: 10578520]
- Harris-White ME, Chu T, Balverde Z, Sigel JJ, Flanders KC, Frautschy SA. Effects of transforming growth factor-beta (isoforms 1–3) on amyloid-beta deposition, inflammation, and cell targeting in organotypic hippocampal slice cultures. *J Neurosci.* 1998; 18(24):10366–74. [PubMed: 9852574]
- Hastings NE, Simmers MB, McDonald OG, Wamhoff BR, Blackman BR. Atherosclerosis-prone hemodynamics differentially regulates endothelial and smooth muscle cell phenotypes and promotes pro-inflammatory priming. *Am J Physiol Cell Physiol.* 2007; 293(6):C1824–33. [PubMed: 17913848]

- Heissig B, Hattori K, Friedrich M, Rafii S, Werb Z. Angiogenesis: vascular remodeling of the extracellular matrix involves metalloproteinases. *Curr Opin Hematol*. 2003; 10(2):136–41. [PubMed: 12579040]
- Hiraoka N, Allen E, Apel II, Gyetko MR, Weiss SJ. Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. *Cell*. 1998; 95(3):365–77. [PubMed: 9814707]
- Hockel M, Schlenger K, Doctrow S, Kissel T, Vaupel P. Therapeutic angiogenesis. *Arch Surg*. 1993; 128(4):423–9. [PubMed: 7681277]
- Hotary K, Allen E, Punturieri A, Yana I, Weiss SJ. Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3. *J Cell Biol*. 2000; 149(6):1309–23. [PubMed: 10851027]
- Hughes GC, Biswas SS, Yin B, Coleman RE, DeGrado TR, Landolfo CK, Lowe JE, Annex BH, Landolfo KP. Therapeutic angiogenesis in chronically ischemic porcine myocardium: comparative effects of bFGF and VEGF. *Ann Thorac Surg*. 2004; 77(3):812–8. [PubMed: 14992878]
- Ichioka S, Minh TC, Shibata M, Nakatsuka T, Sekiya N, Ando J, Harii K. In vivo model for visualizing flap microcirculation of ischemia-reperfusion. *Microsurgery*. 2002; 22(7):304–10. [PubMed: 12404349]
- Itoh T, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res*. 1998; 58(5):1048–51. [PubMed: 9500469]
- Itoh Y, Ito N, Nagase H, Evans RD, Bird SA, Seiki M. Cell surface collagenolysis requires homodimerization of the membrane-bound collagenase MT1-MMP. *Mol Biol Cell*. 2006; 17(12):5390–9. [PubMed: 17050733]
- Itoh Y, Seiki M. MT1-MMP: a potent modifier of pericellular microenvironment. *J Cell Physiol*. 2006; 206(1):1–8. [PubMed: 15920734]
- Johnson PC, Mikos AG, Fisher JP, Jansen JA. Strategic directions in tissue engineering. *Tissue Eng*. 2007; 13(12):2827–37. [PubMed: 18052823]
- Josko J, Mazurek M. Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis. *Med Sci Monit*. 2004; 10(4):RA89–98. [PubMed: 15039660]
- Lee YS, Jang HS, Kim JM, Lee JS, Lee JY, Li Kim K, Shin IS, Suh W, Choi JH, Jeon ES, et al. Adenoviral-mediated delivery of early growth response factor-1 gene increases tissue perfusion in a murine model of hindlimb ischemia. *Mol Ther*. 2005; 12(2):328–36. [PubMed: 16043101]
- Lehr HA, Leunig M, Menger MD, Nolte D, Messmer K. Dorsal skinfold chamber technique for intravital microscopy in nude mice. *Am J Pathol*. 1993; 143(4):1055–62. [PubMed: 7692730]
- Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A*. 2001; 98(1):31–6. [PubMed: 11134512]
- Madeddu P. Therapeutic angiogenesis and vasculogenesis for tissue regeneration. *Exp Physiol*. 2005; 90(3):315–26. [PubMed: 15778410]
- Neth P, Ries C, Karow M, Egea V, Ilmer M, Jochum M. The Wnt signal transduction pathway in stem cells and cancer cells: influence on cellular invasion. *Stem Cell Rev*. 2007; 3(1):18–29. [PubMed: 17873378]
- Neumann U, Kubota H, Frei K, Ganu V, Leppert D. Characterization of Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂, a fluorogenic substrate with increased specificity constants for collagenases and tumor necrosis factor converting enzyme. *Anal Biochem*. 2004; 328(2):166–73. [PubMed: 15113693]
- Nickerson MM, Song J, Shuptrine CW, Wieghaus KA, Botchwey EA, Price RJ. Influence of poly(D,L-lactic-co-glycolic acid) microsphere degradation on arteriolar remodeling in the mouse dorsal skinfold window chamber. *J Bone Miner Res*. 2008
- Nicosia RF, Bonanno E, Smith M. Fibronectin promotes the elongation of micro-vessels during angiogenesis in vitro. *J Cell Physiol*. 1993; 154(3):654–61. [PubMed: 7679680]
- Ono M, Kuwano M. Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. *Clin Cancer Res*. 2006; 12(24):7242–51. [PubMed: 17189395]

- Penault-Llorca F, Bibeau F, Arnould L, Bralet MP, Rochaix P, Sabourin JC. [EGFR expression in colorectal cancer and role in tumorigenesis]. *Bull Cancer*. 2005; 92(Spec no):S5–11. [PubMed: 16387663]
- Pepper MS. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscler Thromb Vasc Biol*. 2001; 21(7):1104–17. [PubMed: 11451738]
- Rucker M, Laschke MW, Junker D, Carvalho C, Schramm A, Mulhaupt R, Gellrich NC, Menger MD. Angiogenic and inflammatory response to biodegradable scaffolds in dorsal skinfold chambers of mice. *Biomaterials*. 2006; 27(29):5027–38. [PubMed: 16769111]
- Sithu SD, English WR, Olson P, Krubasik D, Baker AH, Murphy G, D'Souza SE. Membrane-type 1-matrix metalloproteinase regulates intracellular adhesion molecule-1 (ICAM-1)-mediated monocyte transmigration. *J Biol Chem*. 2007; 282(34):25010–9. [PubMed: 17591781]
- Sodek KL, Ringuette MJ, Brown TJ. MT1-MMP is the critical determinant of matrix degradation and invasion by ovarian cancer cells. *Br J Cancer*. 2007; 97(3):358–67. [PubMed: 17609667]
- Stetler-Stevenson WG. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest*. 1999; 103(9):1237–41. [PubMed: 10225966]
- Takeshita S, Zheng LP, Brogi E, Kearney M, Pu LQ, Bunting S, Ferrara N, Symes JF, Isner JM. Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest*. 1994; 93(2):662–70. [PubMed: 7509344]
- Timur AA, Driscoll DJ, Wang Q. Biomedicine and diseases: the Klippel-Trenaunay syndrome, vascular anomalies and vascular morphogenesis. *Cell Mol Life Sci*. 2005; 62(13):1434–47. [PubMed: 15905966]
- van Hinsbergh VW, Koolwijk P. Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. *Cardiovasc Res*. 2008; 78(2):203–12. [PubMed: 18079100]
- Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell*. 1998; 93(3):411–22. [PubMed: 9590175]
- Wieghaus KA, Capito SM, Anderson CR, Price RJ, Blackman BR, Brown ML, Botchwey EA. Small molecule inducers of angiogenesis for tissue engineering. *Tissue Eng*. 2006; 12(7):1903–13. [PubMed: 16889520]
- Wieghaus, KA.; Capito SM.; Brown, ML.; Botchwey, EA. Novel angiogenic compounds for targeted drug delivery. In: Laurencin, CT.; Botchwey, EA., editors. *Nanoscale Materials Science in Biology and Medicine*. Warrendale, PA: Materials Research Society; 2005. p. 357-61.
- Wieghaus KA, Gianchandani EP, Brown ML, Papin JA, Botchwey EA. Mechanistic exploration of phthalimide neovascular factor 1 using network analysis tools. *Tissue Eng*. 2007; 13(10):2561–75. [PubMed: 17723106]
- Wieghaus KA, Gianchandani EP, Paige MA, Brown ML, Botchwey EA, Papin JA. Novel pathway compendium analysis elucidates mechanism of pro-angiogenic synthetic small molecule. *Bioinformatics*. 2008a; 24(20):2384–90. [PubMed: 18718940]
- Wieghaus KA, Nickerson MM, Petrie Aronin CE, Sefcik LS, Price RJ, Paige MA, Brown ML, Botchwey EA. Expansion of microvascular networks in vivo by phthalimide neovascular factor 1 (PNF1). *Biomaterials*. 2008b
- Will H, Atkinson SJ, Butler GS, Smith B, Murphy G. The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation. Regulation by TIMP-2 and TIMP-3. *J Biol Chem*. 1996; 271(29):17119–23. [PubMed: 8663332]
- Wolf K, Wu YI, Liu Y, Geiger J, Tam E, Overall C, Stack MS, Friedl P. Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. *Nat Cell Biol*. 2007; 9(8):893–904. [PubMed: 17618273]

APPENDIX

Supplementary Tables 1–7, describing the Ingenuity Pathway Analysis (IPA)-generated networks at each of the timepoints.

Supplementary Table 8, describing genes with significant fold changes after PNF1 treatment.

Supplementary Figure 1, showing the Ingenuity Pathway Analysis (IPA)-generated networks at each of the timepoints.

Supplementary Figure 2, showing numbers of gene products differentially expressed at any one, two, three four, or five of the seven timepoints.

Supplementary Figure 3, showing the similarities in top-scoring Ingenuity Pathway Analysis (IPA) networks at 2 and 48 h.

Figure 1. Characterization of genetic regulation by PNF1 at 1–48 h

Panel (a) illustrates the numbers of up- and down-regulated gene transcripts at each of the seven time-points evaluated. Panel (b) describes the networks generated by Ingenuity Pathways Analysis (IPA) for these gene transcripts.

Figure 2. Identification of significantly differentially regulated genes APP, EGR-1, and MT1-MMP

Numbers of gene transcripts that were differentially regulated at multiple timepoints were evaluated; APP, EGR-1, and MT1-MMP (MMP14) were differentially regulated at a maximum of five timepoints (no genes were differentially regulated at any six or all seven timepoints). Panel (a) plots the time course of fold changes for these three genes across the seven timepoints evaluated. Data points in which the gene product has significant fold change regulation stimulated by PNF1 (p -values calculated via the Wilcoxon signed rank test using the GeneChip Operating Software (GCOS; Affymetrix, Santa Clara, CA) are filled in black; insignificant data points are unfilled. Any p -value < 0.003 (as outputted by GCOS) is indicative of an increase in gene expression intensity (PNF1-treated versus control),

whereas a p -value > 0.997 is indicative of a decrease in gene expression intensity. Panel (b) summarizes regulatory and binding roles as well as overall cellular functionality for these three genes, as characterized in the literature.

Figure 3. *In vitro* validation of MT1-MMP (MMP14) activity using a fluorogenic substrate assay HMVEC cultures were stimulated with vehicle control or 30 μ M PNF1 for 1, 2, 4, and 8 h. In addition, 5 μ M FS-6, a fluorogenic substrate capable of measuring relative MMP activity, was added to the medium. Fluorescence observations were consistent with microarray data. Specifically, at 1 h and 4 h post-supplementation, relative FS-6 activity in PNF1-treated cultures was increased as compared with control ($*p < 0.05$), which is consistent with microarray-based findings. Likewise, after 2 h, PNF1-treated samples demonstrated 81% of the FS-6 fluorescence observed in control samples ($*p < 0.05$), which is also consistent with microarray experiments that suggested MT1-MMP mRNA was down-regulated by approximately -1.75 -fold (or 0.57 -fold) in PNF1-treated cultures after 2 h. Only at 8 h did the results of the fluorogenic assay differ from the microarray data, but there was no statistical significance in these data. These data demonstrate that PNF1 does specifically and significantly modulate MT1-MMP activity in HMVECs.

Figure 4. *In vitro* validation of MT1-MMP (MMP14) activity using a collagen invasion assay HMVEC cultures in 2.5 $\mu\text{g/mL}$ collagen gels were stimulated with 30 μM PNF1, vehicle control, tissue inhibitor of metalloproteinase-1 or -2 (TIMP-1 or TIMP-2), or a combination of these factors for 24 h. Panel (a) illustrates representative images of HMVECs stimulated with vehicle control, PNF1, PNF1 + TIMP-1, or PNF1 + TIMP-2 (scale bar = 50 μm). In accordance with previous experiments performed in Matrigel (see (Wieghaus et al. 2006)), PNF1 stimulated more cell elongation and more cell-cell associations than vehicle control samples. This activity was attenuated in response to 2.5 $\mu\text{g/mL}$ TIMP-2, an inhibitor of MT1-MMP, but not in response to 2.5 $\mu\text{g/mL}$ TIMP-1, an inhibitor of soluble MMPs but not membrane-bound MT1-MMP (Sodek et al. 2007). These results are quantified in panel (b), which depicts the number of multiple-cell associations in each culture type after 24 h. PNF1

increased invasion as compared with control samples. Cell invasion stimulated by PNF1 through collagen was not significantly inhibited by 2.5 µg/mL TIMP-1; in contrast, the number of cell-cell associations significantly decreased ($p < 0.05$) with the addition of TIMP-2 at the same concentration.

Figure 5. Potential signaling pathways associated with PNF1 mechanism

PNF1 modulates expression of APP, EGR-1, and MT1-MMP/MMP14, as shown in Figure 3. Here, the process of angiogenesis is diagrammed, including endothelial proliferation and migration associated with extracellular matrix (ECM) degradation. The means by which APP, EGR-1, and MT1-MMP regulate vascular remodeling are also shown, demonstrating mechanisms by which PNF1 may exert its angiogenic effect.

Table 1
Details of RT-PCR conditions for each primer pair

The forward and reverse oligonucleotide sequences for each primer pair, as well as the annealing temperature, are specified for each gene product validated with RT-PCR (Anderson et al. 2008; Hastings et al. 2007; Wieghaus et al. 2007).

Gene product	Dir.	Oligonucleotide sequences (5'- 3')	Annealing temperature (°C)	Reference
18S	F:	CGG CTA CCA CAT CCA AGG AA	60.0	Wieghaus et al. 2007
	R:	AGC TGG AAT TAC CGC GGC		
TGFβ1	F:	CTA CTA CGC CAA GGA GGT CAC	57.7	Wieghaus et al. 2007
	R:	TTG CTG AGG TAT CGC CAG GAA		
CD36	F:	TGC TGT CCT GGC TGT GTT TG	60.0	Anderson et al. 2006
	R:	GCT GCT GTT CAT CAT CAC TTC C		
FN1	F:	AAC GAT CAG GAC ACA AGG AC	61.0 – 63.0	
	R:	CCT CTC ACA CTT CCA CTC TC		
KLF2	F:	GCA CCG CCA CTC ACA CCT G	63.1 – 65.0	Hastings et al. 2007
	R:	CCG CAG CCG TCC CAG TTG		

Table 2
Validation of microarray data with RT-PCR

Fold changes measured by microarray and RT-PCR analyses are compared for four representative genes. In general, there was consistency between the two different experimental platforms.

Gene product	Time (h)	Microarray	RT-PCR
TGFβ1	1	1.07	1.03
	2	1.15	N/A
	4	-1.15	-3.78
	8	1.15	N/A
	16	1.07	3.76
	48	1.07	1.97
CD36	1	1.41	1.79
	2	1.62	3.89
	4	-2.83	-1.05
	8	1.62	1.06
	16	-1.52	-2.45
	48	-2.30	N/A
FN1	1	-1.15	1.16
	2	1.23	1.16
	4	1.00	-1.05
	8	1.07	1.03
	16	-1.15	-1.12
	48	1.23	2.11
KLF2	1	-3.52	-1.01
	2	-2.68	-13.74
	4	-1.40	1.73
	8	1.46	1.74
	16	-2.85	-1.58
	48	1.24	2.34

Table 3
Key characteristics of the genetic networks in HMVECs at 1, 2, 4, 8, 16, 24, and 48 h post-stimulation with PNF1

For each timepoint, the regulated networks generated by Ingenuity Pathway Analysis (IPA) are represented by the top molecular and cellular functions associated with the regulated genes, the gene transcripts with the top magnitudes of fold change (PNF1-treated versus control samples), and the gene transcripts with the highest numbers of interconnections to others in the network. (See the Appendix for a complete version of this table, as well as comprehensive network diagrams across all timepoints.)

Time (h)	Top molecular and cellular functions	Top fold changes	Top connected genes in networks			
	Function	P-value	Gene name	Fold change	Gene name	% Connections in IPKB Database
1	gene expression	1.78E-5 - 2.30E-2	fibronectin 1 (FN1)	22.63	amyloid beta (A4) precursor protein (APP)	12 3.0
	cell death	5.45E-5 - 2.30E-2	syndecan 2 (SDC2)	19.7	jun oncogene (JUN)	12 1.8
	cell morphology	8.17E-5 - 2.30E-2	integrin, alpha M (ITGAM)	-11.31	early growth response 1 (EGR1)	10 3.6
2	cell death	9.5E-5 - 4.98E-2	mitogen-activated protein kinase 8 interacting protein 3 (MAPK8IP3)	14.93	caspase 3, apoptosis-related cysteine peptidase (CASP3)	30 4.5
	RNA post-transcriptional modification	2.58E-4 - 4.69E-2	TNF receptor-associated factor 5 (TRAF5)	13.93	catenin (cadherin-associated protein), beta 1 (CTNBNB1)	29 5.5
	cellular assembly and organization	6.15E-4 - 4.11E-2	caspase 2, apoptosis-related cysteine peptidase (CASP2)	-16	jun oncogene (JUN)	29 4.3
	cell death	7.13E-5 - 1.99E-2	hepatocyte growth factor (HGF)	19.7	interleukin 6 (IL6)	16 1.9
4	cell-to-cell signaling and interaction	7.13E-5 - 1.99E-2	heparanase (HPSE)	14.93	hepatocyte growth factor (HGF)	13 3.9
	cellular development	1.64E-4 - 2.06E-2	apoptosis inhibitor 5 (API5)	13.93	amyloid beta (A4) precursor protein (APP)	9 2.3
8	cell morphology	2.30E-5 - 3.25E-2	collagen, type XIV, alpha1 (undulin, COL14A1)	21.11	fibronectin 1 (FN1)	13 3.3
	cellular movement	8.55E-5 - 3.25E-2	interleukin 18 (IL18)	18.38	protein tyrosine phosphatase, non-receptor type 11 (PTPN11)	9 4.4
	cell-to-cell signaling and interaction	1.28E-4 - 3.25E-2	phosphodiesterase 11A (PDE11A)	18.38	catenin (cadherin-associated protein), beta 1 (CTNBNB1)	9 1.7
16	cell morphology	1.64E-4 - 4.85E-2	CD80 molecule (CD80)	-29.86	nuclear receptor co-repressor 1 (NCOR1)	13 10.5

Time (h)	Top molecular and cellular functions	Top fold changes	Top connected genes in networks				
	Function	P-value	Gene name	Fold change	Gene name	# of Connections	% Connections in IPKB Database
24	cellular assembly and organization	2.96E-4 - 3.55E-2	ADAM metalloproteinase domain 2 (fertilin beta, ADAM2)	-39.4	cyclin D1 (CCND1)	11	2.4
	cell cycle	8.06E-4 - 4.90E-2	endothelin receptor type B (EDNRRB)	-84.45	SMAD family member 4 (SMAD4)	9	4.7
	cell death	9.09E-6 - 2.59E-2	ras homolog gene family, member H (RHOH)	11.31	transforming growth factor, beta 1 (TGFB1)	21	2.0
	cellular growth and proliferation	5.92E-5 - 2.62E-2	EPH receptor B2 (EPHB2)	10.56	amyloid beta (A4) precursor protein (APP)	7	1.8
	cellular assembly and organization	7.13E-5 - 2.64E-2	heparanase (HPSE)	-13.93	cell division cycle 2 (CDC2)	7	2.5
	48	cellular function and maintenance	3.78E-5 - 4.48E-2	collagen, type III, alpha 1 (COL3A1)	39.4	mitogen-activated protein kinase 8 (MAPK8)	35
gene expression		8.92E-5 - 4.48E-2	claudin 23 (CLDN23)	18.38	epidermal growth factor receptor (EGFR)	27	5.4
amino acid metabolism		1.14E-4 - 2.82E-2	S100 calcium binding protein G (S100G)	-10.56	vascular endothelial growth factor (VEGF)	27	5.7