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Mechanistic Exploration of Phthalimide Neovascular Factor 1 Using Network Analysis Tools

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Abstract

Neovascularization is essential for the survival and successful integration of most engineering tissues after implantation *in vivo*. The objective of this study was to elucidate possible mechanisms of phthalimide neovascular factor 1 (PNF1), a new synthetic small molecule proposed for therapeutic induction of angiogenesis. Complementary deoxyribonucleic acid microarray analysis was used to identify 568 transcripts in human microvascular endothelial cells (HMVECs) that were significantly regulated after 24-h stimulation with 30 μ M of PNF1, previously known as SC-3–149. Network analysis tools were used to identify genetic networks of the global biological processes involved in PNF1 stimulation and to describe known molecular and cellular functions that the drug regulated most highly. Examination of the most significantly perturbed networks identified gene products associated with transforming growth factor-beta (TGF- β), which has many known effects on angiogenesis, and related signal transduction pathways. These include molecules integral to the thrombospondin, plasminogen, fibroblast growth factor, epidermal growth factor, ephrin, Rho, and Ras signaling pathways that are essential to endothelial function. Moreover, real-time reverse-transcriptase polymerase chain reaction (RT-PCR) of select genes showed significant increases in TGF- β -associated receptors endoglin and beta glycan. These experiments provide important insight into the pro-angiogenic mechanism of PNF1, namely, TGF- β -associated signaling pathways, and may ultimately offer new molecular targets for directed drug discovery.

INTRODUCTION

The Clinical Goals of Tissue Engineering are to repair, replace, or regenerate damaged or lost tissues in the body. In recent years, exciting new advances in stem cell technologies, scaffold biomaterials, and growth factor therapies have foreshadowed a new era in regenerative medicine as tissue engineering draws nearer to widespread clinical use. However, significant challenges must be addressed before the promise of tissue engineering is fully realized.

One particularly important challenge for the advance of tissue-engineering applications is the development of effective therapies to promote neovascularization of engineered tissues.^{1–5} The formation of new blood vessels via angiogenesis is critically important for many clinical applications,^{6–11} particularly tissue engineering applications in which significant nutritive and metabolic demands are created. Currently, research strategies for therapeutic neovascularization have emphasized focal delivery of growth factors^{9–13} and the investigation of their role in the regulation of microvascular pattern formation. However, although peptide-based growth factors have enormous promise for developing effective strategies in tissue engineering, several potential drawbacks have been reported, such as high costs associated with recovery and purification of recombinant proteins and susceptibility to aggregation and degradation.^{12,14} Processes such as sterilization, solubilization, and storage that are required to deliver the proteins efficiently in the clinical setting may also exacerbate the latter effects.¹⁵ Moreover, size, hydrophilicity, and physical and chemical lability can adversely affect their pharmacokinetic and pharmacodynamic properties.¹⁶ Indeed, clinical trials have highlighted potential alterations in efficacy due to mode of growth factor delivery.¹⁷

More recently, the importance of naturally occurring small-molecule regulators of angiogenesis has been increasingly recognized. For example, insights obtained from lysophospholipid growth factor receptor knockout animals showing severe abnormalities in vascular development have fueled significant interest in lysophospholipid growth factors such as sphingosine-1-phosphate for use as an angiogenic agent to treat ischemic diseases.^{18–20} In other reports, dimethyl-oxalylglycine^{21,22} and other naturally derived alkaloids, terpenes, and phenolic compounds have been discovered that inhibit tissue oxygen homeostatic agent hypoxia inducible factor-1 inactivation, resulting in a pleiotropic angiogenic response. These and other studies signal the advent of new small-molecule strategies for induction and therapeutic manipulation of angiogenesis.

Our group recently reported the development of the first synthetic small-molecule inducer of angiogenesis, phthalimide neovascular factor (PNF1, formerly known as SC-3–149).²³ *In vitro* evaluation of the compound demonstrated that it is effective in enhancing proliferation, survivability, and *in vitro* capillary network formation by endothelial cells.²³ Preliminary *in vivo* studies also showed early-stage angiogenic response induced by the drug in rat mesenteric windows. These studies suggested that PNF1 and its analogues might serve as promising new angiogenic agents for targeted drug delivery and therapeutic angiogenesis in tissue engineering.

The focus of this study was to elucidate candidate mechanisms of the drug using transcriptional regulatory network analysis. Transcriptional profiling is a valuable tool for elucidation of mechanisms underlying many biological pathways.²⁴ Genomic signatures that arise from applying complementary deoxyribonucleic acid (cDNA) microarray technologies can be used to discover unknown mechanisms of action of small molecules and drugs, to predict drug efficacy, and to identify potential new therapeutics.²⁴ We performed a comparative cDNA microarray analysis of gene products isolated from cultures of human microvascular endothelial cells stimulated with PNF1 and those stimulated with a vehicle control.

We used network tools to characterize the transcriptional and molecular level effects of PNF1 stimulation of microvascular endothelial cells in an effort to identify potential mechanistic targets of the drug. Examination of data using network analysis tools instead of evaluation of single-gene perturbation revealed key regulatory events in signaling that may not have led to significant changes in messenger ribonucleic acid (mRNA) expression of any particular gene product. We demonstrate that 24-h stimulation of microvascular endothelial

cells with PNF1 results in concerted regulation of many gene products that have known regulatory effects on vascular remodeling. The primary networks of gene products revealed in our analysis are centered upon the transforming growth factor-beta (TGF- β) signaling pathway that has known effects on angiogenesis. Identifying a mechanism for the angiogenic effects of PNF1 is essential for appropriate utilization of these PNF1 and related compounds as vascular therapies, and will further understanding of how small molecule inducers of angiogenesis might impact the fields of tissue engineering and regenerative medicine.

MATERIALS AND METHODS

Cell culture

Human microvascular endothelial cells (HMVECs) (Cambrex, Walkersville, MD) were cultured in endothelial growth medium 2-microvascular (bulletkit, BioWhittaker, Walkersville, MD) supplemented as directed with 5% fetal bovine serum. Cells were cultured on tissue culture plates (Nalge Nunc, Rochester, NY) at 37°C in a humidified chamber with 5% carbon dioxide.

RNA isolation

HMVECs (passage 9) were plated at 2.5×10^4 cells/cm² on tissue culture plastic (Nalge Nunc, Rochester, NY) and grown to confluence. After confluence, medium was refreshed, and 30 μ M PNF1 or 0.6% dimethyl sulfoxide vehicle control was added to the sample. After 24 h of supplementation, total RNA from the cultures was isolated using an RNeasy kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol.

Gene array and analysis

RNA samples were prepared for hybridization using the GeneChip One-Cycle Target Labeling and Control Reagents kit (Affymetrix, Santa Clara, CA). Expression profiles were created using Human Genome U133 Plus 2.0 arrays (Affymetrix) with approximately 47,000 transcripts and variants, including 38,500 well-characterized human gene transcripts. Expression data were analyzed using GeneChip Operating Software (Affymetrix), which calculates signal intensities and detection calls along with their related *p*-values for each probe set representing a gene transcript. The software automatically assigned the change between the experimental and baseline conditions to a standard category (increase, marginal increase, no change, marginal decrease, or decrease), depending on the *p*-value (calculated using the Wilcoxon signed rank test); 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. Data will be deposited into the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>).

Network and gene ontology analyses

The gene products identified as significantly differentially regulated (up- or downregulated) according to microarray analysis were used for further network and gene ontology analyses. For these analyses, Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com), coupled with the Ingenuity Pathways Knowledge Base (IPKB), was employed. IPA has been used recently to study genetic pathways and mechanisms of cell and tissue cultures, those stimulated with small molecules,^{25–28} toxic elements,²⁹ and radiation therapies³⁰ or those associated with pathologies.³¹ The IPKB offers genome-wide coverage of more than 23,900 mammalian genes; millions of pathway interactions manually curated from the literature; and representation of biological specificity, including species, location, mutations, and experimental conditions.

A data set containing gene transcript identifiers and fold-change information (of PNF1-stimulated samples over that of controls) was uploaded into IPA. The software was used to overlay a subset of these genes eligible for generating networks onto a global molecular network developed from information contained in the IPKB. The subset of genes, called focus genes, consisted of genes with associated molecular interaction information within the IPKB. Networks of these focus genes were then generated based on their connectivity, with each network composed of a maximum of 35 gene products, as currently limited by the IPA software for computational reasons (physical memory). These networks were ranked according to a score representing the probability that each isolated network of genes could be achieved by chance alone. A score greater than 3 was considered significant; with this score, there is a 1 in 1,000 chance that the focus genes in the network were isolated because of random chance (scores of ≥ 3 have a 99.9% confidence level of not being generated by random chance alone).

Additionally, IPA was used to characterize suggested molecular and cellular functions for the data set as a means of determining key processes in the cell samples that were regulated by treatment with the drug. The IPA tool isolated 244 gene products eligible for this type of functional analysis. The significance associated with a function is a measure of how likely it is that genes from the data set under investigation participate in that function. The significance is expressed as a *p*-value, which is calculated using the right-tailed Fisher exact test. In this method, the *p*-value is calculated by comparing the number of user-specified genes of interest that participate in a given function or pathway with the total number of occurrences of these genes in all functional or pathway annotations stored in the IPKB.

IPA generated graphical representations of the molecular relationships between genes and gene products to further investigate the significantly regulated networks. Genes or gene products are represented as nodes, and the biological relationship between any two nodes (e.g., phosphorylation, transcription) is represented as an edge (line). At least one reference from information stored in the IPKB supports each edge. The intensity of the node color indicates the degree of up- (red) or downregulation (green). Nodes are displayed using various shapes that represent the functional class of the gene product. Edges may be displayed with various labels that describe the nature of the relationship between the nodes (e.g., P for phosphorylation or T for transcription).

Quantitative real-time PCR

cDNA (from 0.01 $\mu\text{g}/\mu\text{L}$ of RNA solution) was synthesized with a Bio-Rad iScript kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Five μL of the subsequent cDNA template was added to a reaction well containing 10 μL each of 0.5- μM solutions of forward and reverse gene-specific primers (Table 1) and 25 μL of iQ SYBR Green Supermix (Bio-Rad), including SYBR green, deoxyribonucleotide triphosphates, Taq enzyme, and reaction buffer. PCR reactions were performed using an iCycler thermal cycler (Bio-Rad); an initial denaturing step was performed at 9°C, before 40 cycles of 95°C for 10 s, $x^\circ\text{C}$ for 30 s, and 72°C for 45 s, where *x* is the specific annealing temperature of the primer set. Reactions were performed for the following genes: TGF- β 1, activin-like kinase 1 (ALK1), ALK5, TGF- β receptor II (TGF- β RII), beta glycan, CD105, CD36, epidermal growth factor receptor (EGFR), and acidic fibroblast growth factor 1 (FGF1). The 18S gene was used as a housekeeping gene.

All primer sequences and primer-specific annealing temperatures can be found in Table 1. All assays were performed in triplicate. Data are reported using means $\pm$ standard errors of the mean.

RESULTS

Comparative microarray analysis

To investigate the transcriptional regulation of HMVECs by synthetic small-molecule inducer of angiogenesis PNF1, comparative microarray analysis was performed on control- and drug-treated cultures after 24 h of stimulation. Differential expression between control- and PNF1-stimulated culture samples was found in 568 gene transcripts on the array. The GeneChip Operating Software characterized these differentially expressed gene transcripts as increased, decreased, or marginally increased or decreased; results can be found in Table 2. Approximately 57% of the significantly regulated genes were downregulated, and almost 18% were marginally regulated, as determined according to the corresponding *p*-values as described in the Materials and Methods section. Table 2 also reports the regulation of the 264 genes available for network analysis and the 244 functional analysis genes (see “Network and gene ontology analyses” below). The percentages of genes whose expression level increased or decreased across these subsets of genes were consistent.

Observation of the most significant changes in single gene expression, as measured according to microarray, after 30- μ M PNF1 stimulation of HMVECs revealed that the drug substantially upregulated insulin-like growth factor (IGF) binding protein 6 (IGFBP6) and angiopoietin-like factor 4 (ANGPTL4) after 24 h. The pro-angiogenic⁴² IGF family is known to promote migration and tube formation of vascular endothelial cells,⁴³ and IGFBP6 is upregulated in response to hypoxia in cultured endothelial cells.⁴⁴ Similarly, ANGPTL4 is an important modulator of vascular permeability⁴⁵ that is produced during ischemia to promote angiogenesis.⁴⁶ These molecules may ultimately prove to be viable candidates for signaling pathways that control the pro-angiogenic effects of PNF1 in HMVECs.

Network and gene ontology analyses

We employed network tools to examine the coordinated perturbation of entire networks of gene products by PNF1. The microarray-identified genes were further analyzed using IPA to investigate the nature of their biological interaction. Of the 568 genes, 264 focus genes were identified as the subset of gene products with associated molecular interaction information in the IPKB. Exploration of the regulation of the remaining gene products may be performed in future experiments. The focus genes were then mapped to genetic networks as generated by the IPA tool, using known signaling pathways and molecular interactions from the literature to describe functional relationships between the gene products. IPA then associated the networks with known biologic pathways; 16 networks of 35 genes were identified as significant, in that more of the identified genes were present than could be expected by chance (score > 3). See Table 3 for a comprehensive listing of the identified networks. All of the significantly regulated networks were interconnected; that is, each of the networks can be linked to all of the other PNF1-regulated networks via gene product interactions in the IPKB. The networks were arranged according to decreasing score and percentage incorporation of focus genes from the microarray data. For example, 100% of the genes tabulated in network 1 are focus genes, and the network was assigned a score of 56, meaning that there is a 1 in 10⁵⁶ chance that the collection of genes were regulated together by chance.

The Functions feature of the IPA suite was used to identify particular high-level cellular processes associated with microvascular stimulation of PNF1 after 24 h. Table 4 details each cellular process and its relative significance or the probability that the included genes are involved with the particular function. The most significantly activated cellular processes included cell-to-cell signaling and interaction, cellular assembly and organization, cellular movement, cellular growth and proliferation, and cell signaling, all of which are integral to

the process of angiogenesis. PNF1 has previously been demonstrated to significantly promote endothelial cell proliferation, migration, and tubule formation *in vitro*,²³ and here we report that the genes regulated by this small molecule as identified according to this analysis support our previous findings.

Networks 1 and 2 were identified as having substantially higher scores associated with their regulation and larger incorporation of focus genes than the other networks; hence, we chose to focus on these networks for further analysis. Clustering diagrams of each network can be found in Figure 1A and B, respectively. As described previously, nodes (i.e., gene products) are displayed as various shapes, and the edges between them (i.e., biological relationships) are shown as arrows connecting them. The IPA tool included 5 of the gene products in network 2 (Fig. 1B) in the network, but they were not perturbed, as reported by the original microarray results. It is possible that a concurrent signaling mechanism counter-regulated these non-perturbed gene products or that their individual gene regulation was subtle but sufficient to effect a change in downstream regulation.

Networks 1 and 2 were then joined via known signaling connections between the 2 pathways for identification of candidate signaling pathways for PNF1 (Fig. 2), because there was a large overlap between the gene sets of the 2 networks. IPA identified 26 molecular interactions between the 2 genetic networks, as denoted by blue connectors in Figure 2. The gene product with the most molecular interactions is TGF- β 1. TGF- β signaling has been shown to play a significant role in the development and function of the cardiovascular system and vascular network formation.^{47–51} The TGF- β Rs include the serine-threonine kinase type I (including ALK1 and ALK5) and TGF- β RIIs,^{52,53} and the TGF- β RIIIs extracellular betaglycan⁵⁴ and endoglin (CD105).^{55,56} Additionally, there is mounting evidence that the TGF- β s can signal through multiple other pathways.⁴⁷

Signaling pathways emanating from TGF- β 1 in Figure 2 that might represent the molecular mechanism for the *in vitro* angiogenic effects of PNF1 on HMVECs are presented in Table 5 and illustrated in Figure 3. Each of these signaling pathways are well known to enhance cellular proliferation and viability significantly or give other molecular cues for stimulation of angiogenesis that parallel results reported after stimulating microvascular endothelial cells with PNF1 as an initial determination of drug function.²³ These include molecules integral to the thrombospondin, plasminogen, FGF, EGF, ephrin, Rho, and Ras signaling pathways that are important to endothelial function. As Table 5 demonstrates, each of these signaling pathways and associated gene products has been characterized in the literature as having functions that are implicated in angiogenesis or cellular functions related to the angiogenic process, including maintenance of cell viability and adaptation to hypoxia, stimulation of endothelial proliferation and migration, degradation of surrounding extracellular matrix, increased production of other angiogenic factors, and vascular network formation and remodeling. For example, TGF- β 1 has been found to decrease expression of CD36,⁵⁷ a receptor for thrombospondin and mediator of its anti-angiogenic activity;^{58–61} significant downregulation of CD36 by PNF1 stimulation may inhibit angiostatic signaling by thrombospondin.

Additionally, TGF- β 1 is known to decrease expression of cell division cycle 2^{89,90} (cdc2, also known as p34, a ribosome-binding protein), a trigger for the apoptosis signaling cascade,^{62,63} in various cell types. PNF1 downregulated cdc2 and, hence, may be responsible for observed increases in endothelial cell viability in nutrient-deprived conditions, as has been shown previously.⁶³ Cdc2 has also been found to bind to FGF1,⁶⁴ a well-known angiogenic growth factor⁷⁵ with increased expression after PNF1 exposure. TGF- β 1 also stimulates expression of RhoB (a Rho family GTPase),^{66,67} which PNF1 also upregulated. This intracellular signaling molecule, among other functions, controls stage-

specific survival of endothelial cells during vascular development via Akt survival signaling pathway;⁶⁸ therefore, RhoB activation by PNF1 may also contribute to its effects on *in vitro* cell viability.

Another potential mechanism for the angiogenic effects of PNF1 on microvascular endothelial cells occurs through EGFR. TGF- β 1 has been implicated in the activation and phosphorylation of EGFR,^{69,70} an angiogenic growth factor.^{71,72} This study demonstrates that upregulation of EGFR after stimulation with PNF1 may induce stimulation of ephrin receptor B2, which is well known to affect proper vascular development and angiogenesis^{74,75} via Ras activation.⁷³ TGF- β 1 protein also has known interactions with hairy/enhancer-of-split related with YRPW motif 1 (HEY1), a binding protein; literature findings suggest that supplementing various types of cells with TGF- β 1 increases mRNA expression of HEY1,⁷⁶ although our data indicate that HEY1 was downregulated after stimulation with PNF1. This may have important implications for the exact mechanism of action, but downregulation of HEY1 may prove to be important to the angiogenic process, because overexpression is known to block normal vascular network formation in mouse embryos.⁷⁷ High-mobility group A1 (HMGA1), a DNA binding protein that is involved in apoptotic signaling⁷⁸ and expression of nitric oxide synthase,⁷⁹ an important signaling molecule in angiogenesis,⁸⁰ also interacts with TGF- β 1.⁷⁹ Our data show that expression of HMGA1 was upregulated, which is consistent with PNF1 effects on cell death and *in vitro* tubule formation. Additionally, TGF- β 1 upregulates expression of non-muscle myosin polypeptide 9 (MYH9)⁶⁷ gene product; this protein fragment has been demonstrated to be necessary for angiogenic nucleolin signaling.⁸¹

According to our microarray data and literature findings, TGF- β 1 upregulates transcription and expression of urokinase plasminogen activator, also known as uPA,⁸² which further activates tissue-type plasminogen activator, or tPA.⁸³

Both types of plasminogen activators are highly expressed during vascular sprouting and angiogenesis, because they contribute to the degradation of extracellular matrix that is necessary for new vessel growth.^{84–86} Finally, according to the IPA tool, TGF- β 1 indirectly (through mitogen-activated protein kinase (MAPK8) regulates activity of SMT3 suppressor of mif two 3 homolog 1 (yeast) (SUMO1),⁸⁷ an enzyme that is implicated in apoptosis signaling in the cardiovascular system. Our data indicate that SUMO1 was downregulated, which parallels *in vitro* cell viability data for PNF1, and that promyelocytic leukemia (PML) protein, a downstream transcription regulator, was highly downregulated. PML is integral to TGF- β 1 apoptosis signaling,⁸⁸ so its regulation may also contribute to decreases in apoptosis seen after PNF1 supplementation in microvascular cell cultures.

To validate results obtained from the microarray experiment, as has been reported in the literature,⁹¹ TGF- β 1, CD36, EGFR, and FGF1 from networks 1 and 2 were selected for quantitative RT-PCR analysis. As demonstrated previously, TGF- β 1 was the gene product with the highest number of signaling interactions in networks 1 and 2. CD36, EGFR, and FGF1 were selected because of their known effects on angiogenic signaling pathways and their high degree of regulation by the novel drug. Because TGF- β 1 is known to induce its own receptors,⁹² we assayed the 5 known receptors as well. These 9 genes had varying magnitudes of differential expression (Table 6). In 8 of 9 (88%) of the assays performed, a gene product that was upregulated in the microarray experiment was also upregulated when using the PCR method. Although RT-PCR did not validate ALK5 signaling perturbation as measured using microarray, the gene was not significantly regulated in either case, showing only a 1.15-fold change according to RT-PCR compared with a negative change in expression value according to microarray.

Target genes specific to TGF- β 1R signaling have been investigated previously. Lux *et al.*³³ overexpressed ALK1 in HMVEC_s via viral transfection to decouple the transcriptional profile of TGF- β 1-stimulated endothelial cells from that of ALK1-specific signaling. Table 6 presents the expression levels of the TGF- β Rs, as stimulated by low (0.5 ng/mL) and high (4 ng/mL) levels of TGF- β 1 and ALK1 overexpression,³³ and compares them with results for 30- μ M PNF1 obtained via cDNA microarray and real-time RT-PCR analysis. The change in expression of TGF- β 1Rs TGF β R2, ALK1, and ALK5 was unremarkable after 24 h, although it is possible that changes in the expression of these genes may occur at earlier timeframes after stimulation with the drug. Moreover, treatment with PNF1 significantly upregulated the beta glycan and endoglin accessory TGF- β Rs. Endoglin is primarily expressed in angiogenic endothelial cells and is upregulated during hypoxia^{93,94} and promotes ALK1 signaling transduction in proliferating endothelial cells.⁹⁵ Conversely, beta glycan has been found to inhibit angiogenesis in human cancer cells.⁹⁶ The 2 analogues are known to form complexes with one another on human microvascular endothelial cells.⁹⁷ It is thought that perhaps the interaction between endoglin and beta glycan may be necessary for maintenance of positive and negative aspects of TGF- β signal transduction on endothelial cells.⁹⁷

DISCUSSION

The novel small molecule PNF1 has been shown to stimulate angiogenic activity in microvascular endothelial cells *in vitro*, including enhanced proliferation, viability, tube formation, and migration.²³ Here, our data demonstrate that the stimulation of microvascular endothelial cells over 24 h with PNF1 in its most-effective *in vitro* concentration (30 μ M) produces a significant change in the regulation of their genetic networks and related cell-signaling processes. Although single-gene regulation may prove important in understanding the drug's mechanism of action, a focus on the concerted regulation of larger networks of genes by an angiogenic agent might lend more valuable insights, because the differential expression of many gene products that are integral to the angiogenic process may be subtle. For example, this type of network analysis could elucidate potential side effects of this or any drug before testing in animal or clinical studies. Thus, we chose to employ network tools to examine the influence of PNF1 on transcriptional regulation of microvascular cells on a larger scale than single-gene product analysis allows.

After 24 h of stimulation, PNF1 perturbs a number of signaling pathways that are known to regulate the angiogenic process. Figure 2 demonstrates that the most-targeted regulation by the drug (networks 1 and 2) occurred through a TGF- β 1-related signaling pathway. It is important to note that TGF- β 1 is a well-characterized growth factor, and therefore more potential interactions may be curated in the database for this molecule than other gene products in the networks. However, statistical examination of the networks validates the importance of the coordinated perturbation of these genes, which share known relationships to TGF- β 1 signaling. Network analysis methods employed in this study reveal that the statistical probability of the coordinated regulation of gene products listed in networks 1 and 2 unrelated to PNF1 stimulation is low (1 in 10^{56} and 1 in 10^{42} , respectively), but although drug supplementation clearly initiated the TGF- β 1-centered networks of genes products, the differential regulation of TGF- β 1 itself after 24 h was unremarkable—only 1.3-fold according to cDNA microarray analysis. Use of network analysis tools instead of examination of single genes allowed for identification of key regulatory events in signaling that may not have led to significant changes in mRNA expression of any particular gene product. The statistical power afforded by this method of analysis permitted us to focus on distinct sets of gene products that drug stimulation most highly regulated. Additionally, association between the pro-angiogenic effects of PNF1 and a particular pathway (TGF- β signal transduction) allows for the investigation of related gene products, such as receptors,

that were not classified in regulated networks at a particular time point but are integral to signaling events that define the mechanism of action of PNF1.

Our microarray data, compared with that of Lux *et al.*,³³ gives no definitive indication of which (if any) TGF- β signaling mechanism our drug initiates. Moreover, it is known that the effect of TGF- β signaling of endothelial cells varies during microvascular network remodeling, although application of PNF1 after 24 h upregulated the 2 accessory TGF- β Rs beta glycan and endoglin. The interplay between the 2 molecules is integral to the balance of pro- and anti-angiogenic signaling in microvascular cells; therefore, future examination of the genetic regulation of these receptors by the drug, including their differential regulation at earlier time points, may provide important insight into the mechanism of action of PNF1.

PNF1 also significantly affected the regulation of signaling molecules that are not directly involved with known TGF- β R signaling. For example, PNF1 may alter expression of extracellular matrix proteases or promotion of monocyte recruitment and associated secretion of pro-angiogenic cytokines.⁹⁸ Members of the Ras and MAPK pathways;⁴⁷ including K-ras and MAPK4 and MAPK8, were also identified in significantly regulated networks 1 and 2 (Fig. 2). In addition, PNF1 decreased the expression of CD36, the receptor for anti-angiogenic thrombospondin.^{58–60}

In conclusion, we used network analysis tools to elucidate what global networks of transcriptional regulation mediated by PNF1 produce effects in microvascular endothelial cells that are consistent with angiogenic stimulation.²³ However, angiogenesis is a complex process that requires a number of cell types and a myriad of complicated, somewhat poorly understood signals, and therefore the implications of cell responses assessed using standard *in vitro* culture methods on *in vivo* angiogenesis are indirect. Nevertheless, our studies show that network tools are particularly effective in elucidating coordinated perturbation of entire networks of gene products by PNF1, which would not be possible through the examination of single-gene regulation alone. Moreover, we identified a well-known signal transduction pathway (TGF- β) regulated by PNF1 after 24 h and examined several smaller groups of genes that may ultimately reveal the drug's mechanism of action. We believe that delivery of PNF1 is a potentially effective strategy to improve tissue repair outcomes, where revascularization is known to be a limiting step to successful healing.

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FIG. 1.

Clustering diagrams of Ingenuity Pathway Analysis-generated networks (**A**) 1 and (**B**) 2. Shaded genes were identified using microarray analysis, and others were associated with the regulated genes using network analysis. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). At least one reference from information stored in the Ingenuity Pathways Knowledge Base supports each edge. The intensity of the node shading indicates the degree of regulation. Nodes are displayed using various shapes that represent the functional class of the gene product. Color images available online at www.liebertpub.com/ten.

FIG. 2.

Networks 1 and 2 as merged by the Ingenuity Pathway Analysis tool according to known molecular associations between the gene products of interest. Bold connecting lines signify associations that join the 2 networks. A legend for node and edge shapes, describing the function of each gene product as well as their interactions with one another, is shown at the right. Color images available online at www.liebertpub.com/ten.

FIG. 3.

Potential signaling pathways induced by 30 μ M phthalimide neovascular factor 1 after 24 hours, emanating from transforming growth factor- β . Solid lines represent a known direct relationship; dashed lines indicate a relationship that may be direct or indirect (as included in the Ingenuity Pathways Knowledge Base). Color images available online at www.liebertpub.com/ten.

Table 1
Details of Real-Time Polymerase Chain Reaction Conditions for Each Primer Pair

Gene product	Oligonucleotide sequences (5' - 3')	Primer annealing temperature (°C)	Reference
<i>TGF-β Receptors</i>			
TGFB1	(F): CTACTACGCCAAGGAGGTAC (R): TTGCTGAGGTATCGCCAGGAA	57.7	32
ALK1	(F): GCAACCTGCAGTGTTCATC (R): CGGATCTGCTCGTCCAGCAC	59.1	33
ALK5	(F): TCGTCTGCATCTCACTCAT (R): GATAAATCTCTGCCTCAG	51.7	34
TGFBRII	(F): AGCAACTGCAGCATCACCTC (R): TGATGTCTGAGAAAGATGTC	54.7	35
betaglycan	(F): ACATGGATAAGAACGATTTCAGC (R): AACGCAATGCCCATCACGGTTAG	57.8	36
endoglin	(F): GCTGGATGAGCGGAGCTCCCTGCTG (R): CACAGGCTGAAGGTCAAAATGGACTG	65.6	37
<i>Proteins Implicated in Angiogenesis</i>			
CD36	(F): TGGGAAAGTCACCTGGCAGCAT (R): TGCAATACCTGGCTTTTCTCA	55.7	38
EGFR	(F): AACACCTGGTCTGGAAGTAC (R): ACACCAGTTGAGCAGGTACTG	56.7	39
FGF1	(F): CAAACTCTCTCTAGTACCAACGGG (R): TTGCTTTCTGGCCATAGTGAGTCCG	60.0	40
<i>Housekeeping Control</i>			
18S	(F): CGGCTACCACATCCAAAGGAA (R): GCTGGAAATACCGGGCT	57.8	41

Table 2

Numbers of Characterized Genes Regulated Upon Treatment with 30- μ M Phthalimide Neovascular Factor 1, The Maximum Effective Dose *In Vitro*,²³ After 24H

Expression change	Transcripts, n (of the 568 differentially- regulated)	Transcripts, n (of the 244 eligible for functional analysis)	Transcripts, n (of the 264 eligible for network analysis)
Decrease	324	130	144
Marginal decrease	60	26	27
Marginal increase	41	16	17
Increase	143	72	76

Changes between experimental and control conditions were assigned as follows: increase, marginal increase, no change, marginal decrease, or decrease depending on the *p*-value calculated by the software by using the Wilcoxon signed rank test. *P*-value ranges for each call were 0.0000–0.0025, 0.0025–0.0030, 0.0030–0.9970, 0.9970–0.9975, and 0.9975–1.0000, respectively. Expression changes are reported for the 568 differentially regulated genes, the 244 genes eligible for functional analysis, and the 264 genes eligible for network analysis.

Table 3
Genetic Networks in Human Microvascular Endothelial Cells Stimulated with 30- μ M Phthalimide Neovascular Factor 1

Network	Genes in network [†]	Score [‡]	Focus genes (%)	Functions
1	AGRN, APP, CD36, CD47, CD151, CDC2, COL8A1, CST3, DLX2, FGF1, GCLC, GCLM, HEY1, IGFBP6, IL1RN, INHBA, INHBE, ITGA4, ITGA6, ITPR1, JAG1, KPNAB6, LRPIB, LYN, MYH9, PDLIM4, PDLIM7, PLAT, PLAU, PTPRC, SERPINE2, SPTAN1, SPTEN1, TGFB1, TSPAN4	56	100	Cell-to-cell signaling and interaction, cellular movement, cell death
2	AKAP9, APBB1IP, CASR, CD44, CTTN, DNMI1, EGFR, EPHB2, ERO1L, F2RL1, FUT8, GAB1, HMGAI, HSPH1, IRE4, ITSN1, KRAS, MAP4K4, MAPK8, NRAS, PAMCL, PDE4D, PIK3CD, PML, RECK, RET, RHOB, RHOC, RRAS, SENP1, SH2D3C, SOD2, SUMO1, TIMP2, TXN	42	86	Cellular movement, cell death, cellular growth and proliferation
3	ALS2CR8, ATP5A1, ATP5B, ATP5C1, ATP5D, ATP5E, ATP5O, BDNF, C6ORF182, CAMK2D, CBX5, CDC25C, DPPA4, GADD45A, HMG3, JMJD5, KC1D1, MLH1, MSH2, MSH3, MSH6, MSRA, MUTHY, NFYB, NFYC, PCNA, PITPNM3, POLH, POLI, RFC3, SEPT7, SERBP1, SH3GL1, SYN1, ZNF587	17	49	DNA replication, recombination, and repair; cell death, connective tissue development and function
4	ARG1, C6ORF108, CD19, CDCA7, CEBPA, CTSD, CYHP2, FKBP4, FTH1, FUBP1, FXR2, GATA2, GGH, IREB2, ITGAX, KLF2, MYC, PGK1, PPIA, PPID, PRDX2, PRDX3, PRKACB, PTPRC, RBMS1, RFX3, RPL30, SIAHBP1, SLC25A5, SNRPN, TFR, TUBB2A, VDAC2, YY1, ZFYVE20	16	46	Viral function, cellular function and maintenance, small molecule biochemistry
5	ADORA2B, AEBP2, BAK1, BCHE, BCKDHA, CCL19, CD44, COLQ, CXCL13, DBT, DOK2, FLT3, FLT3LG, GNAI3, GPD2, HIPK2, HPSE, HSPG2, IER3, IL7, IPLA2(GAMMA), LTB, MCL1, NR6A1, OPRM1, PEX11A, PRSS23, RGS14, RGS16, RGS20, SDC4, SOX4, TM4SF1, TNF, TNFSF11	14	43	Cellular development, immune and lymphatic system development and function, cellular movement
6	ACTN4, ALDOA, ATP6V0E, ATP6V1E1, BAK1, BASP1, BMPRIA, BMX, CASP3, CASP4, COL4A1, EAF1, ELL, EVI1, HCFCIR1, HMGAI, IL2RB, JUP, LMNB1, MYCN, PARG, PARK2, PODXL, RFC3, RUFY2, SLC9A3R2, SRY, STAT1, SUMO1, TEK, TNFRSF10B, TUBB, VIL2, WAC, WTI	14	43	Cancer, cell death, reproductive system disease
7	ADCYAP1, ADD3, ARHGDIA, ARHGDIB, ARHGDIG, AZIN1, BCR, CD44, CDC42, CDC42BPB, COMMD1, COMMD2, COMMD4, COMMD10, COMMD5 (includes EG:28991), COPE, COPG, COPG2, COPZ1, EFNBI, GNRHR, IQGAP1, MAPK1, PEX5, PEX10, PEX12, PEX5L, POMC, RAB8B, RHOF, RHOF, SACMIL, SCN7A, SCNN1B, SCNN1G, VAV2	14	43	Cell signaling, cellular assembly and organization, genetic disorder
8	ALG5, ASF1A, ATG5, CCND1, CDK8, CRSP4, CRSP5, CRSP6, CRSP7, DDX11, DMXL1, EPAS1, HATI, HCAP-D3, HCAP-H2, L1UZP5, MED12, MED28, MYST2, MYST3, MYST4, NUT2, PCAF, PML, RBL1, RHOF, SMC1L1, SMC2L1, STAG1, SURB7, TAF1, TCEB1, TCEB3,	14	43	Gene expression, cell cycle, cellular assembly and organization

Network	Genes in network [†]	Score [‡]	Focus genes (%)	Functions
THRAP6, VHL				
9	ABII, ADIPOQ, ANGPTL4, APOA5, G6PD, GPX3, HOOK1, INS1, KRIT1, LAMA2, LAMA3, LAMB1, LAMB3, LAMC2, LAMC3, LIFR, LPL, MIR16, POLD4, RABAC1, RALA, RAP1A, RGS2, SIM1, SIRT1, SLC12A3, STX6, STX7, STX1B2, STXBP3, TYROBP, VAMP2, VAMP8, VPS18, VTI1A	14	43	Dermatological diseases and conditions, genetic disorder, cellular assembly and organization
10	APIG2, DCP1B, EXOSC1, EXOSC2, EXOSC3, EXOSC6, EXOSC7, EXOSC8, EXOSC9, HNRPA1, KPNB1, LARP1, MOC33, NCKAP1, NUP98, PABPC1, PAN3, RBMX, SAPS3, SARIB, SEC13L1, SEC31L1, SFRS10, SFRS12, SMAD3, SPTBN1, TERF1, TGFBR1, TNKS2, UCHL5, USP52, XRN1, XRN2, YWHAG, ZFP36	14	43	Cellular movement, hair and skin development and function, RNA post-transcriptional modification
11	ATR, BAI3, BCR, CHEK1, DDC, EEF1E1, EGR3, GSTP1, IFNA2, KNTC1, MEI, MLLT4, NOX4, NRXN2, ODC1, PLAGL1, PPM1D, PRKCD, PRKDCBP, PRKDC, PTPRA, PVRL1, PVRL2, PVRL3, PVRL4, RCHY1, SORBS1, THRAP4, THRB, TP53, UBE2B, YY1, ZNF148, ZW10, ZWILCH	13	40	Cell cycle, DNA replication, recombination, and repair, cellular compromise
12	CDKN1A, CHAF1B, CLEC7A, CTH, E2F4, F2, GATA2, HCLS, HIPK2, HSPA9B (includes EG-3313), IER2, IER3, IL4, KAL1, LAMP2, LGALS1, MGLL, MRPS10, ORC1L, ORC3L, ORC4L, ORC5L, ORC6L, PML, ROCK2, SLC30A5, SLC3A2, SLC7A11, SMC2L1, SMC4L1, SRM, SXX2IP, TEC, TMPO, TP73L	13	40	DNA replication, recombination, and repair, gene expression, cell cycle
13	AKT1, AREG, ARHGEF1, ARL4A, CAPNS1, CCL19, CCNA2, CD44, CREBBP, CSPG2, CTNNB1, DKK1, FOXD3, FXYD5, GPX1, HBEFG, IL3, ITGAX, JUB, JUN, MST1, MST1R, MUC6, MUT, NCOR2, NME3, PLAGL1, PML, PRKDC, PTMS, RPL10, SIM2, SND1, TSC22D1, WNK1	13	40	Gene expression, cellular growth and proliferation, cellular development
14	ACY1, ATP1A4, CD40LG, CIASI, CXCL5, DMD, DTNA, DTNB, DUSP5, EB12, EIF4E, EPRS, FGF2, GLG1, HDAC2, IL1B, JTV1, KARS, LARS, MARS, MEFV, NFKB1B, PDCD2, PML, PPP1R14A, PYCARD, QARS, RARS, SCYE1, SGC8, SNTG1, TTRAP, YY1, ZNFEN1A2, ZNFEN1A3	13	40	Cardiovascular system development and function, cell morphology, RNA post-transcriptional modification
15	BNIP3, CAPN1, CAPN3, CAPN6, CARHSP1, CCK, CD44, CDC37, CLCA2, DES, DHRS7, DSG2, ELF3, ERBB2, ERRF1, ETFB, G3BP, G6PD, GRB7, IL6R, ITGB4, ITGB4BP, LIFR, MATK, MME, MSN, NRGI, PAFAH1B1, PDHA1, PLEC1, TGFBR1, TPD52, TPD52L1, VCL, VIL2	12	37	Cellular movement, cancer, cellular growth and proliferation
16	ADAM15, ADD2, AR, CD36, CTNND1, EPHA4, EPS8, EPS8L2, ETV5, EVL, FGG, FLOT1, FLOT2, FYN, HBEFG, HELLS, HSPB6, INSR, ITGB3, MAPK3, NCOR2, PPAP2B, PRACA, PTPRA, PTPRF, RP2, RPS6KA6, SCAP1, SEZ6L2, SMAD4, SORBS1, SOS1, STUB1, TCF7L2, TRIM33	12	37	Cell cycle, posttranslational modification, cellular movement

[†] Bold genes were identified by microarray. Others were not significantly regulated, and were introduced by the IPA tool using known molecular associations contained within the IPKB.

[‡] Score>3 was considered significant.

Table 4

Molecular and Cellular Functions Significantly Regulated by Phthalimide Neovascular Factor 1 in Endothelial Cells

Molecular and cellular function	P value	Genes, n
Cell-to-cell signaling and interaction	1.14e-4 to 4.87e-2	36
Cellular assembly and organization	4.41e-4 to 4.87e-2	43
Cellular movement	9.19e-4 to 4.87e-2	26
Cellular growth and proliferation	1.29e-3 to 4.87e-2	19
Cell signaling	2.05e-3 to 4.87e-2	6
Molecular transport	2.05e-3 to 4.87e-2	17
Small molecule biochemistry	2.05e-3 to 4.87e-2	21
Cell cycle	2.36e-3 to 4.87e-2	28
Cell morphology	3.46e-3 to 4.87e-2	14
Cellular development	3.46e-3 to 4.87e-2	22
Cell death	3.46e-3 to 4.87e-2	30
Cellular function and maintenance	3.46e-3 to 4.87e-2	21
Post-translational modification	3.46e-3 to 4.87e-2	2
Cellular compromise	8.86e-3 to 4.87e-2	22
Gene expression	1.33e-2 to 4.87e-2	12
Cellular response to therapeutics	1.41e-2 to 1.41e-2	3
Amino acid metabolism	1.81e-2 to 4.87e-2	5
Drug metabolism	2.14e-2 to 4.87e-2	7
Free radical scavenging	2.71e-2 to 3.93e-2	8
Nucleic acid metabolism	4.22e-2 to 4.87e-2	4
Energy production	4.87e-2 to 4.87e-2	1
Lipid metabolism	4.87e-2 to 4.87e-2	8
Protein degradation	4.87e-2 to 4.87e-2	2
Protein synthesis	4.87e-2 to 4.87e-2	1
Vitamin and mineral metabolism	4.87e-2 to 4.87e-2	3

Potential Signaling Pathways, and Their Phenotypic Effects, Associated With Phthalimide Neovascular Factor 1 in Human Microvascular Endothelial Cells

Table 5

Potential pathway	Downstream effect	References
TGFB1, CD36	decreased activity of thrombospondin (angiogenesis inhibitor)	57, 58, 59, 60, 61
TGFB1, CDC2, FGF1	decreased apoptosis signaling, increased angiogenesis	62, 63, 64, 65
TGFB1, RHOB	increased EC survival in developing vasculature, cellular adaptation to hypoxia, contributes to vascular sprout morphology	66, 67, 68
TGFB1, EGFR, RRAS, EPHB2	proliferation, decreased inhibition of vascular network remodeling, vascular sprouting	69, 70, 71, 72, 73, 74, 75
TGFB1, HEY1	decreased blockage of vascular network formation	76, 77
TGFB1, HMGAI	decreased apoptosis, increased NOS expression	78, 79, 80
TGFB1, MYH9	increased angiogenic action of nucleolin	67, 81
TGFB1, PLAU, PLAT PLAU /PLAT	very highly expressed during endothelial sprouting and angiogenesis; degradation of extracellular matrix	82, 83, 84, 85, 86
TGFB1, MAPK8, SUMO1, PML	proliferation, increased growth, increased Smad signaling, decreased apoptosis	87, 88

Real-Time Confirmation of Microarray Data, and an Overview of Phthalimide Neovascular Factor 1–Regulated Gene Expression of Transforming Growth Factor Beta (TGF- β) Receptors Compared with Those of Low and High TGF- β 1 Concentrations and Constitutively Active Activin-Like Kinase 1 Receptor Signaling After 24H

Table 6

	Average differential gene expression				
	30 μ M PNF1, RT-PCR ($\pm$ SEM)	30 μ M PNF1, Microarray	0.5 ng/mL TGF- β 1 ³³	4 ng/mL TGF- β 1 ³³	ALK1 Overexpression ³³
ALK1	-1.39 ($\pm$ 0.03)	-1.07	1.01	1.01	1.40
ALK5	1.15 ($\pm$ 0.90)	-1.15	1.07	1.43	1.36
betaglycan	3.11 ($\pm$ 1.03)	2.30	nt	nt	nt
endoglin	1 ($\pm$ 1.34)	3.03	1.04	1.22	2.36
TGFBR2	-1.08 ($\pm$ 0.99)	-1.07	-1.03	-1.01	1.26
FGF1	2.42 ($\pm$ 1.07)	5.6			
EGFR	2.61 ($\pm$ 1.18)	8			
CD36	-1.20 ($\pm$ 1.83)	-13.90			
TGFB1	3.77 ($\pm$ 1.02)	1.3			

From Lux *et al.*³³

Standard errors are reported in parentheses.

nt=not tested