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Transcriptional Activation of Placental Growth Factor by the Forkhead/Winged Helix Transcription Factor FoxD1

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Summary

Stromal-epithelial interactions play an important role in renal organogenesis [1]. Expression of the forkhead/winged helix transcription factor FoxD1 (BF-2) is restricted to stromal cells in the embryonic renal cortex, but it mediates its effects on the adjacent ureteric bud and metanephric mesenchyme, which fail to grow and differentiate in BF-2 null mice [2]. BF-2 is therefore likely to regulate transcription of factors secreted by stromal cells that modulate the differentiation of neighboring epithelial cells. Here, we used cells with inducible expression of BF-2, combined with microarray analysis, to identify Placental Growth Factor (PlGF), a Vascular Endothelial Growth Factor (VEGF) family member previously implicated in angiogenesis, as a downstream target of BF-2. BF-2 binds to a conserved HNF3β site in the PlGF promoter and activates transcription. PlGF is precisely coexpressed with BF-2, both temporally and spatially, within the developing renal stroma, and it is completely absent in BF-2 null kidney stroma. Addition of PlGF to in vitro kidney organ cultures stimulates branching of the ureteric bud. Our observations indicate that PlGF is a direct and physiologically relevant transcriptional target of BF-2. The contribution of PlGF toward stromal signals that regulate epithelial differentiation suggests novel functions for a growth factor previously implicated in reactive angiogenesis.

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Results and Discussion

Identification of BF-2 Target Genes by Using High-Density Oligonucleotide Microarrays

To identify stromal signals regulated by BF-2, we established tetracyline-regulated expression (tet-off) of wild-type or HA-tagged BF-2 in HeLa cells, a cervical cancer cell line with no expression of the endogenous gene. Western and Northern blot analyses showed tightly regulated induction of BF-2 following tetracycline withdrawal (Figure 1). Total cellular RNA was isolated following growth of cells in the presence of tetracycline or at 6, 12, 18, and 24 hr following drug withdrawal and was used to interrogate high-density microarrays. Genes showing significant >4-fold induction at 24 hr in two independent replicates ($p < 0.001$) and consistent change over the time course studied were identified as potential BF-2 target genes and were subjected to confirmation by Northern blot analysis (Figure 1A).

Three genes were identified by microarray hybridization and were confirmed to be induced by BF-2 by using Northern blotting (Figure 1): PlGF, a member of the VEGF family, which cooperates with VEGF itself in mediating angiogenesis during wound healing and tumorigenesis [3]; heparin binding Epidermal Growth Factor (HB-EGF); and the bHLH protein G0S8. We chose to characterize PlGF in greater detail, since it was induced by BF-2 in all cell lines tested and it encodes a secreted growth factor, whose role in embryonic kidney development could most readily be validated by using in vitro assays.

Induction of PlGF by BF-2

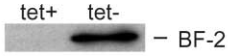
Induction of PlGF rapidly followed that of BF-2, with increased expression within 12 hr after the removal of tetracycline (Figure 1C). Two well-characterized PlGF isoforms, PlGF1 and PlGF2, derived from alternative splicing, have been identified in humans: compared with PlGF1, PlGF-2 contains 21 additional amino acids at its C terminus that confer heparin binding activity [4]. Mouse cells express only one transcript, homologous to PlGF-2. RT-PCR analysis of BF-2-inducible HeLa cells demonstrated comparable induction of both PlGF-1 and PlGF-2 (data not shown).

To confirm that PlGF was also induced by BF-2 in a physiologically relevant cell type, we established tetracycline-regulated expression of BF-2 in RSTEM cells, a rat cell line derived from undifferentiated renal mesenchyme at E12.5 [5]. Consistent with its metanephric mesenchymal origin, RSTEM cells do not express endogenous BF-2. Expression of BF-2 in RSTEM cells also led to prompt induction of endogenous rat PlGF (Figure 1B). The other BF-2 target genes identified in HeLa cells, HB-EGF and G0S8, are already expressed at high levels in RSTEM cells, and no further induction was observed following BF-2 expression.

Activation of the PlGF Promoter by BF-2

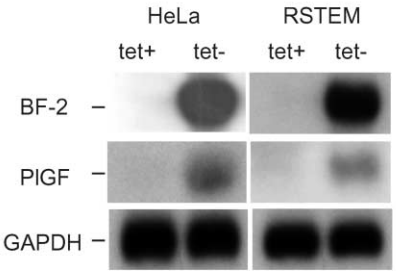
To determine whether PlGF constitutes a direct transcriptional target of BF-2, we cloned its putative up-

A



Accession Number	Description	Array Hybridization Signal tetracycline withdrawal (hrs) 0*	6	12	18	24*	Fold change by Array	Fold change by Northern
X54936	placental growth factor	21.1	-1.5	28	65.4	395.4	+19	>10
M60278	heparin-binding EGF	25.75	31.4	22.2	37.3	325	+12.6	>10
L13463	G0S8	134.95	108.3	120.7	305.8	545.2	+4.0	4

B



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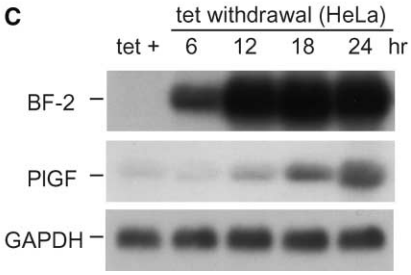


Figure 1. Induction of PlGF mRNA by BF-2

(A) Summary of genes found to be upregulated by microarray analysis and confirmed by Northern blotting following inducible expression of BF-2 in HeLa 24-43 cells. The array hybridization signal is shown for RNA extracted 0, 6, 12, 18, and 24 hr following tetracycline withdrawal (an asterisk denotes average values from duplicate experiments). The fold change from 0 to 24 hr is calculated for array hybridization and Northern blotting results. (Upper panel) Inducible expression of BF-2, demonstrated by Western blot analysis of HeLa 24-19 cells grown in the presence or absence of tetracycline (+tet or −tet).

(B) Representative Northern blots showing induction of Placental Growth Factor (PlGF) coincident with that of BF-2, 24 hr following the withdrawal of tetracycline in HeLa 24-43 and in the rat kidney precursor RSTEM cells (GAPDH; loading control).

(C) Time course of PlGF induction following BF-2 expression in HeLa 24-43 cells grown in the presence of tetracycline or at sequential intervals following drug withdrawal.

stream regulatory region into the promoterless luciferase vector, pGL3. Luciferase activity was examined following transient cotransfection of the reporter construct and a CMV-driven BF-2 expression vector into HeLa cells. A reporter construct containing a 3.5 kb genomic fragment (−1 to −3459) upstream of the PlGF translational start site was activated ~13-fold by BF-2. Progressive deletion of this region indicated the presence of a major BF-2-responsive element within a 250 bp fragment, between −1653 and −1400. The reporter construct containing this fragment was activated ~19-fold by BF-2 (Figures 2A and 2B). Deletion of this region significantly suppressed BF-2 induction of the reporter.

Electrophoretic mobility shift assays (EMSA) demonstrated specific binding of bacterially produced BF-2 protein to the 250 bp fragment; this binding allows further mapping of the binding site (Figure 2C). BF-2 is known to bind a similar DNA consensus sequence as its close homolog, the forkhead/winged helix transcription factor HNF3β, although no transcriptional targets have been identified [6]. A single putative HNF3β binding site is present within the BF-2-responsive region (−1483 to −1502). Indeed, EMSA showed that BF-2 protein binds to a genomic fragment containing the wild-type sequence, but not to one in which the consensus binding sequence had been mutated (Figure 2D). Deletion of this element shows a dramatic reduction in transactivation, although some persistent activation by BF-2 suggests the possibility of additional responsive elements. Taken together, these observations suggest that BF-2 can bind directly to the HNF3β consensus sequence in the PlGF promoter and can thus mediate transcriptional activation.

Coexpression of PlGF and BF-2 in Developing Kidney Stroma

To determine whether the induction of PlGF by BF-2 in cultured cells reflects a physiologically significant interaction, we used RNA in situ hybridization to examine mouse embryonic kidneys at days E12.5 and E14.5 for the expression pattern of BF-2 and PlGF. As previously reported, BF-2 is expressed in a population of stromal cells surrounding the nephrogenic mesenchyme and ureteric bud in E12.5 kidneys (Figures 3A and 3B) [2]. Remarkably, PlGF is expressed at a high level in the same ring of cells surrounding the metanephric mesenchyme and the ureteric bud (Figures 3E and 3F). Low levels of PlGF are also present in some mesenchymal cells that do not express BF-2. In E14.5 kidneys, the nephrogenic zone is limited to the outer edge of the developing kidney, and the epithelial cells derived from the mesenchyme have undergone a series of morphological changes, forming comma- and S-shaped bodies and some early glomeruli. At this stage, BF-2 and PlGF are coexpressed in the cortical stroma that surrounds the developing kidney (Figures 3C and 3G). The develop-

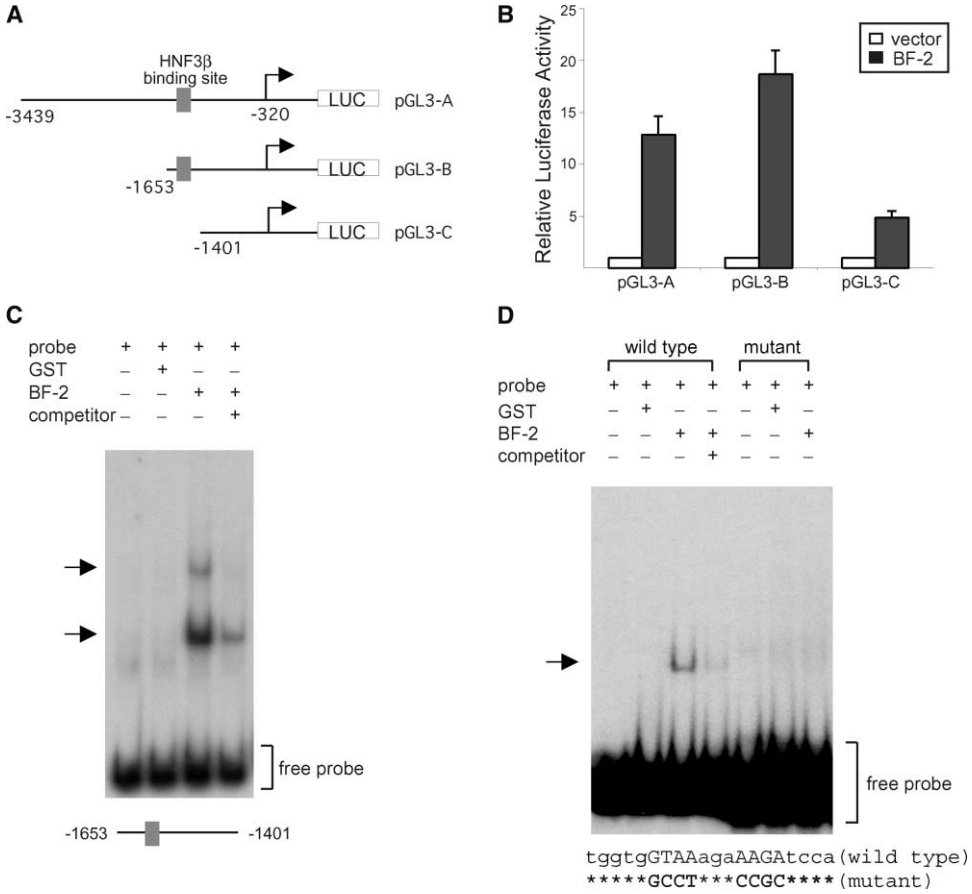


Figure 2. Transcriptional Activation of the PlGF Promoter by BF-2

(A) A schematic representation of promoter-less luciferase reporters containing fragments of the human PlGF promoter. The nucleotide (nt) position of the HNF3β binding site (box) and the transcriptional start site (arrow) are shown with respect to the first ATG.

(B) Fold induction of PlGF reporters (0.05 μg) by cotransfected CMV-BF-2 (0.15 μg) in HeLa cells. Renilla plasmid (0.01 μg) was cotransfected to standardize transfection efficiency, and equal amounts of total DNA were used in each transfection. Standard deviation is derived from at least three independent experiments.

(C) Electrophoretic mobility shift assay (EMSA) analysis, using GST-BF-2 protein incubated with the minimal BF-2-responsive region of the PlGF promoter (−1653 to −1401 nt), identified by luciferase reporters with progressive genomic deletions. The arrows indicate the specific protein:DNA complexes, effectively competed by the addition of unlabeled double-stranded oligonucleotide (50×).

(D) EMSA analysis of BF-2 binding to the wild-type, but not to the mutant HNF3β-responsive element.

ment of glomeruli is accompanied by invasion and proliferation of endothelial cells. PlGF is also present in developing and mature glomeruli, and its presence suggests that it also contributes to recruiting endothelial precursor cells into the glomerular vascular cleft (Figure 3G). In developing glomeruli, as in some mesenchymal cells, expression of PlGF is presumably regulated by factors other than BF-2. In contrast to the restricted expression of PlGF in the differentiating kidney, its homolog VEGF is expressed throughout the nephrogenic mesenchyme in E12.5 kidneys, but only weakly in the ureteric epithelium (Figures 3I and 3J). By E14.5 kidneys, VEGF expressed primarily in the developing glomeruli and tubular structures (Figure 3K). Thus, BF-2 and PlGF are unique in their precise temporal and spatial coexpression in the embryonic renal stroma.

To determine whether BF-2 expression is required for the expression of PlGF in the embryonic renal stroma, we analyzed BF-2 null kidneys by using RNA in situ hybridization (Figure 3D). BF-2(−/−) kidneys show defects in branching of the ureteric bud and in the differentiation of condensed mesenchyme. However, the distribution and amount of stroma is comparable to that of wild-type kidney [2]. Remarkably, PlGF expression is undetectable in the kidney stroma of BF-2(−/−) embryos (Figure 3H); this finding indicates that expression of PlGF in embryonic kidney stroma is dependent upon the presence of BF-2. Low levels of PlGF expression are present in some mesenchymal cells, where it is presumably regulated by factors other than BF-2. The expression of VEGF is not changed in BF-2(−/−) kidneys (Figure 3L).

In examining the expression patterns of the PlGF receptors Flk-1, Flt-1, and Neuropilin-1 in the developing kidney (see the Supplemental Results), we found a high level of Neuropilin-1 expression within the embryonic ureteric epithelium and nephrogenic mesenchyme, whereas Flk-1 and Flt-1 are restricted to vascular struc-

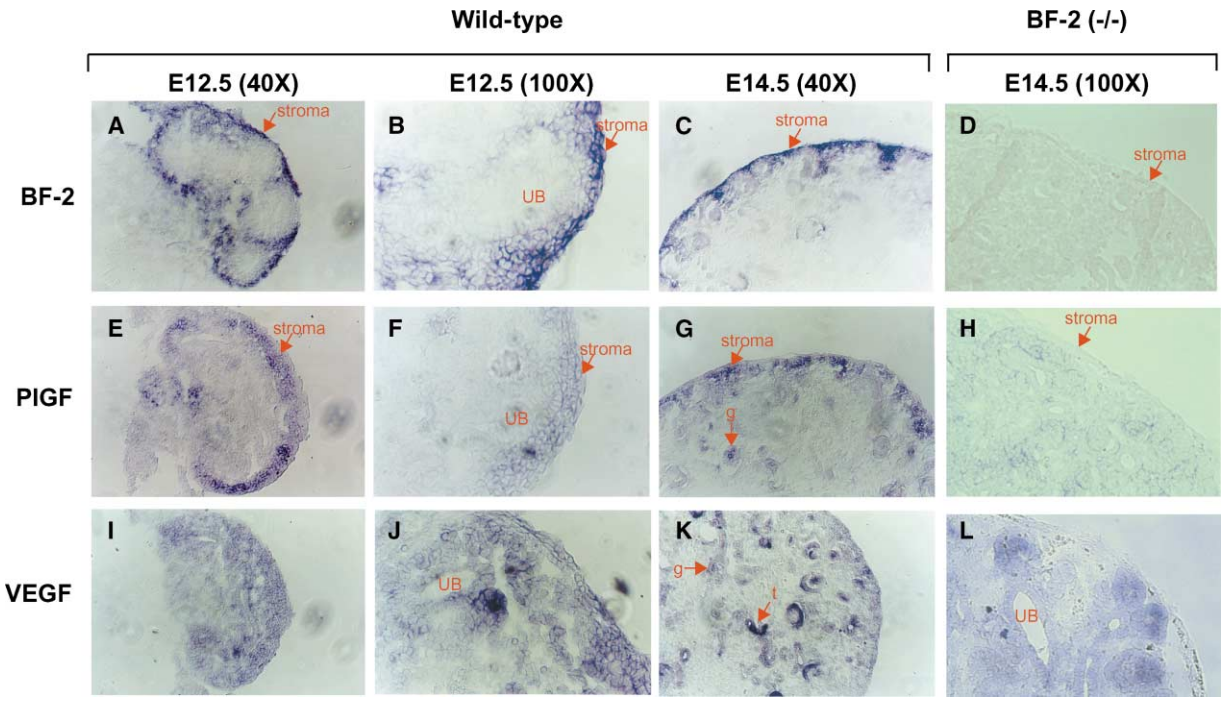


Figure 3. Expression of BF-2, PlGF, and VEGF in Developing Mouse Kidney

(A) RNA in situ hybridization, demonstrating expression of BF-2 transcript in a population of stromal cells surrounding the E12.5 kidney.
(B) Higher magnification, showing that BF-2 is expressed specifically in the cells surrounding the ureteric bud (UB) and condensing mesenchyme. UB and mesenchyme are identified by staining for Wnt-11 and Wnt-4, respectively (not shown).
(C) At day E14.5, BF-2 expression is restricted to the cortical stroma of the kidney.
(D) Absent expression of BF-2 in kidney from the mouse knockout.
(E and F) Expression of PlGF in the stromal compartment of the E12.5 kidney parallels that of BF-2.
(G) At day E14.5, PlGF is present, along with BF-2, in the cortical stroma, as well as in developing glomeruli (g).
(H) Absent expression of PlGF in stroma cells of BF-2(−/−) mice (overexposed).
(I) In contrast to PlGF, expression of VEGF mRNA is present diffusely throughout the developing kidney.
(J) High magnification shows VEGF expression in the stroma and nephrogenic mesenchyme.
(K) At day E14.5, VEGF expression is weak in the outer nephrogenic zone, including the stroma, but high in developing glomeruli (g) and renal tubules (t).
(L) Unaltered expression of VEGF in BF-2(−/−) kidneys.

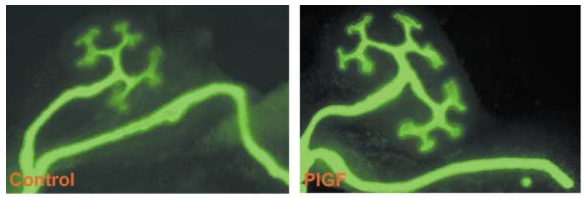
tures. These observations raise the possibility that Neuropilin-1 may mediate a direct effect of PlGF on renal differentiation.

Induction of Ureteric Bud Branching by PlGF in Embryonic Kidney Organ Cultures

Organ cultures of E11.5 mouse kidney offer an invaluable assay of renal differentiation in which to test the direct effect of recombinant growth factors. At this stage, the ureteric bud has just invaginated the mesenchyme, but no branching has occurred. The ureteric bud grows and branches during incubation in vitro, and the metanephric mesenchyme forms aggregates that convert to epithelial cells [7]. To test for functional properties of PlGF in this renal differentiation assay, we examined organ cultures incubated for 40 hr in the presence or absence of recombinant mouse PlGF. Cultures were then stained with an antibody to pan-cytokeratin, which serves as a measure of ureter branching. In multiple experiments, the addition of PlGF increased the number of terminal buds by nearly 40%, from a mean of 16 (±2.9) to 22(±3.1) (n = 24) (Figure 4). This effect is comparable in magnitude to that seen with the addition of GDNF, the major mesenchymal inductive signal for ureteric bud branching (Figure 4) [8].

Conclusions

Here, we have used heterologous cells with tightly regulated, inducible expression of BF-2, together with microarray-based expression profile analysis, to identify PlGF as a specific developmentally regulated transcriptional target of BF-2, likely to contribute to stroma-derived proliferation and differentiation signals. PlGF has previously been linked to pathological and reactive angiogenesis [3]; our observations point to a new role in stimulating branching of the ureteric bud. By virtue of its expression in the ureteric bud, the receptor Neuropilin-1 is a strong candidate to mediate PlGF signaling from stromal cells during ureteric bud branching. In addition, PlGF may also contribute to kidney differentiation indirectly through its effects on endothelial cells carrying the Flt-1 and Flk-1 receptors. Epithelial and vascular differentiation appear to be coordinated in organ cultures [9, 10], and VEGF treatment or hypoxic conditions enhance differentiation of both endothelial and epithelial cells [11, 12]. Thus, PlGF or PlGF/VEGF heterodimers



	Average Number of Branches		
	Control (+/-SD)	Growth Factor (20ng/ml) (+/-SD)	Fold change (%)
Exp. 1 (PlGF, N=8)	16 (+/-2.8)	22.5 (+/-5.2)	40.6
Exp. 2 (PlGF, N=8)	16.8 (+/-3.4)	21.7 (+/-3.3)	29
Exp. 3 (PlGF, N=8)	15 (+/-4.7)	21.4 (+/-4.2)	42.6
Exp. 4 (GDNF, N=4)	10.3 (+/-0.96)	15.3 (+/-0.96)	50
Exp. 5 (PDGF, N=7)	10.1 (+/-1.35)	10.3 (+/-1.11)	1.4

Figure 4. Induction of Ureteric Bud Branching by Recombinant PlGF in Cultured Mouse Kidney Rudiments

(Upper panel) Staining of the ureteric bud network in mouse kidney rudiments, cultured in the absence (control) or presence of 20 ng/ml recombinant mouse PlGF. Paired kidney rudiments were derived from each fetal mouse, cultured in the presence or absence of PlGF, and stained with anti-pancytokeratin, a marker for the Wolfian duct and ureter branches. Representative images are shown. (Lower panel) Quantitation of terminal bud branches was performed for untreated and PlGF-treated cultures by analysis of eight paired rudiments in three independent experiments. The addition of GDNF was used as a positive control (four paired rudiments), and the addition of Platelet-derived growth factor-B (PDGF-B) was used as a negative control (seven paired rudiments).

may stimulate renal epithelial differentiation directly, as well as through the development of capillary networks, which may in turn enhance nephrogenesis. PlGF is unlikely to be the sole mediator of BF-2-induced stromal signals required for epithelial differentiation, since PlGF null mice do not have detectable kidney defects [3]. This may result from functional redundancy within the PlGF family, as well as from the contribution of additional BF-2 transcriptional targets. The aggregate effects of multiple transcriptional targets are presumably required to recapitulate the effects of BF-2 on renal differentiation.

Supplemental Data

Supplemental Data including additional results, the Experimental Procedures, and a figure are available at http://www.current-biology.com/cgi/content/full/13/18/1625/DC1/.

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