Nuclear Receptor COUP-TFII Controls Pancreatic Islet Tumor Angiogenesis by Regulating Vascular Endothelial Growth Factor/Vascular Endothelial Growth Factor Receptor-2 Signaling

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Abstract

The significance of angiogenesis in cancer biology and therapy is well established. In this study, we used the prototypical RIP-Tag model of multistage pancreatic islet tumorigenesis to show that the nuclear receptor COUP-TFII is essential to regulate the balance between pro- and anti-angiogenic molecules that influence the angiogenic switch in cancer. Conditional ablation of COUP-TFII in the tumor microenvironment severely compromised neoangiogenesis and lymphangiogenesis during pancreatic tumor progression and metastasis. We found that COUP-TFII plays a cell-autonomous role in endothelial cells to control blood vessel sprouting by regulating cell proliferation and migration. Mechanistic investigations revealed that COUP-TFII suppressed vascular endothelial growth factor (VEGF)/VEGFR-2 (VEGFR-2) signaling by transcriptionally repressing the expression of VEGFR-1, thereby curtailing a central angiogenic driver of vascular growth. Taken together, our results implicate COUP-TFII as a critical factor in tumor angiogenesis through regulation of VEGF/VEGFR-2 signaling, suggesting COUP-TFII as a candidate target for anti-angiogenic therapy.

Introduction

Tumor angiogenesis, the development of new capillaries from preexisting blood vessels, is indispensable for tumor growth and metastasis (1–3). Without the concomitant growth of new blood vessels, tumors cannot expand beyond a minimal size, invade locally, or metastasize to distant sites (4). It is widely accepted that angiogenesis is regulated by a balance of pro- and anti-angiogenic molecules, when the balance shifts in favor of angiogenesis inducers, and an angiogenic switch activates the normally quiescent vasculature to develop new blood vessels (2, 5). Thus, the “angiogenic switch” during tumorigenesis is recognized as a rate-limiting secondary event that can be effectively targeted as a therapeutic approach to treat cancer (4). Proangiogenic factors, such as vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factors (FGF1 and FGF2), members of the angiopoietin family (Ang1 and Ang2), and platelet-derived growth factor (PDGF), have been shown to induce signaling cascades to increase endothelial cell proliferation, survival, and migration to promote angiogenesis (6). Among them, VEGF is a major mediator of angiogenesis, which transmits its signals primarily through binding to its receptor VEGF receptor-2 (VEGFR-2), a receptor tyrosine kinase, on the surface of blood endothelial cells to promote angiogenesis (7).

Using a rat insulin promoter (RIP) to ectopically express the oncoprotein SV40 T antigen in the β cells of pancreatic islets (RIP1-Tag2; ref. 8), it has been realized that the angiogenic switch plays a crucial role in the development of multistage β-cell carcinogenesis (2, 8). Histopathologic studies have revealed three distinct stages of tumor progression in this model, with hyperplastic lesions observed at 5 weeks of age, followed by the development of angiogetic islets at 7 to 10 weeks of age. A few weeks later, encapsulated adenomas emerge, of which about 25% progress into invasive carcinomas (3).

COUP-TFII, a member of the steroid/thyroid nuclear hormone receptor superfamily (9), plays critical roles in organogenesis (10–14). We showed that COUP-TFII is essential for tumor angiogenesis through regulation of a pericyte-derived paracrine signal (Ang1/Tie2 signaling) that affects the endothelium (15). However, the fact that providing Ang1 can only partially rescue the angiogenic defect suggested that COUP-TFII might regulate other factors to control angiogenesis. To further dissect the underlying mechanism, we took advantage of the RIP1-Tag2 spontaneous pancreatic cancer model to investigate the role of COUP-TFII in pancreatic cancer...
progression. Here, we showed that ablation of COUP-TFIIf in adult mice markedly compromised neoangiogenesis and lymphangiogenesis. We showed that COUP-TFIIf plays an autonomous role in blood endothelial cells to control vessel sprouting by directly regulating VEGFR-1 expression. These findings elucidate the important roles of COUP-TFIIf within the blood endothelial cells to regulate tumor angiogenesis.

Materials and Methods

Animal experiments

RIP1-Tag2 mice were obtained from the National Cancer Institute Mouse Repository, and COUP-TFIIflox/flox and ROSA26Cre-ERT2−/− mice were as previously described (12, 16). Mice were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. The RIP1-Tag2 mice were crossed with ROSA26Cre-ERT2−/−; COUP-TFIIflox/flox mice to generate mice with genotypes of RIP1-Tag2−/−;COUP-TFIIflox/flox and RIP1-Tag2−/−/COUP-TFIIflox/flox, which were then treated with tamoxifen at the age of 5 weeks (17).

Assessment of angiogenic islets, tumor burden, and tumor frequency

Angiogenic islets, which morphologically exhibited a red-dish patch in a white nodular background, were isolated after the mice were perfused with collagenase IV solution (18). In other experiments, tumors were isolated by microdissection, and tumor number and weight were measured.

Cell cultures and reagents

Primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza and cultured according to the supplier's recommendations. Human primary lymphatic endothelial cell (LEC) isolated from neonatal human foreskin as previously described (19) were kindly provided by Dr. Young-Kwon Hong (Keck School of Medicine, University of Southern California, Los Angeles, CA). Small interfering RNA (siRNA) duplexes targeting COUP-TFIIf, VEGFR-1, and Control Non-Target siRNA were purchased from Dharmacon. Cells were transfected with siRNA duplexes by using Oligofectamine (Invitrogen).

HUVEC proliferation and migration analysis

For cell cycle analysis, cells were stained with propidium iodide and analyzed by a FACScan flow cytometer (Becton Dickinson). The number of viable cells was measured using an MTT cell proliferation kit (Promega). Modified Boyden chambers with filter inserts (BD) were used for the Chamber migration assay, and blood endothelial cells were stimulated by VEGF (5 ng/mL).

Western blot analysis

The primary antibodies used in this study were phospho-VEGFR-2 (Tyr1175) and VEGFR-2 (Cell Signaling), COUP-TFIIf (Perseus Proteomics), and VEGFR-1 (Acris Antibody). Tumor lysates were immunoprecipitated with anti–VEGFR-2 overnight and subjected to immunoblotting with anti–phospho-VEGFR-2 antibodies. Protein loading amount was normalized against total VEGFR-2. For VEGFR-1 antibody neutralization assay, anti–VEGFR-1 antibody (R&D) or control IgG was added at a concentration of 250 ng/mL.

HUVEC sprouting assay

HUVEC spheroids of a defined cell number were generated overnight and embedded into collagen gels as described (20). VEGF was used to stimulate cell sprouting at a concentration of 5 ng/mL.

Histology and immunohistochemistry

The primary antibodies used were CD31 (BD), NG2 (Millipore), cleaved caspase-3 (Cell Signaling), phosphorylated histone H3 (Cell Signaling), LYVE-1 (AngioBio), insulin (DAKO), and large T antigen (Calbiochem).

Quantitative real-time reverse transcription-PCR

Gene expression assay was performed using the ABI PRISM 7500 Sequence Detector System (Applied Biosystems), and all mRNA quantities were normalized against 18S RNA or CD31. The primers/probes used in this study were purchased from Applied Biosystems.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were performed using an assay kit (Millipore) and monoclonal anti–COUP-TFIIf (Perseus Proteomics) following the manufacturer's recommendation. The primers used for detection of the VEGFR-1 promoters were 5′-CTGGAAGAAGGATGCTGTTTC-3′ and 5′-CGGGCGGATTTTCTGTC-3′, which were then treated with tamoxifen at the age of 5 weeks (17).

Image quantification and statistical analysis

Images of fluorescent staining were analyzed and quantified using NIH ImageJ. Data represent the mean ± SEM. Statistical significance was calculated by Student’s t test or Fisher’s exact test.

Results

Efficient deletion of COUP-TFIIf in RIP-Tag2 mice

To explore the role of COUP-TFIIf in pathologic vascular processes and tumorigenesis, we inactivated the COUP-TFIIf gene in adult mice using the tamoxifen inducible Cre-recombinase system (ROSA26CRE-ERT2−/−; ref. 16). RIP1-Tag2 mice were crossed with COUP-TFIIflox/flox and ROSA26CRE-ERT2−/− mice to generate genotypes of RIP1-Tag2−/−;COUP-TFIIflox/flox (RIP/+; F/F) and RIP1-Tag2−/−/ROSA26CRE-ERT2−/−/COUP-TFIIflox/flox (RIP/+; Cre+/+; F/F). Both genotypes were subjected to tamoxifen treatment at the age of 5 weeks. COUP-TFIIf was ablated in RIP/+; Cre/+; F/F mice, and RIP/+; F/F mice served as controls. Before assessing the role of COUP-TFIIf in pancreatic β-cell carcinogenesis, we used double immunostaining of COUP-TFIIf and T antigen to evaluate the efficiency of COUP-TFIIf deletion (Supplementary Fig. S1A). T antigen, specifically expressed in β tumor cells, was used to mark the tumor cells. The loss of
COUP-TFII–positive cells in Supplementary Fig. S1A clearly showed that COUP-TFII was efficiently deleted in the mutant mice, which was further confirmed by Western blot analysis (Supplementary Fig. S1B). Because COUP-TFII expression was not colocalized with T antigen expression, it suggests that COUP-TFII is expressed within the tumor microenvironment, but not in the tumor cells. To determine the cell types that expressed COUP-TFII, we used CD31 (endothelial cell) and NG2 (pericyte) as markers. We observed that COUP-TFII was strongly expressed in endothelial cells (Supplementary Fig. S1C, arrow) and pericytes (arrowhead) within the tumor vasculature.

**Involvement of COUP-TFII in the angiogenic switch and tumor progression**

Next, we examined the effects of COUP-TFII depletion on RIP-Tag2 tumorigenesis. Control and COUP-TFII–mutant mice were sacrificed at the age of 12 weeks, shortly before the mice succumbed to hypoglycemia caused by the insulin-expressing tumors. We observed that tumors from COUP-TFII–deficient mice were significantly smaller and appeared much paler compared with the tumors from control mice (Fig. 1A). In accordance, COUP-TFII–mutant animals displayed a significant decrease in aggregate tumor burden in comparison with control animals (Fig. 1A and B).

Pancreatic tumors in the RIP1-Tag2 model can be classified into three types according to established criteria (21): encapsulated islet tumors (IT), microinvasive carcinomas (IC-1), and highly invasive carcinomas (IC-2). It have well-defined margins and frequent fibrous capsules and IC-1 have focal regions of invasion, whereas IC-2 have wide fronts of invasion that prominently intermingled with the surrounding acinar tissue. To evaluate whether loss of COUP-TFII would affect tumor malignance, we performed a histologic analysis to compare the abundances of the various stages of tumors from control and mutant mice. Figure 1C shows that COUP-TFII–deficient mice displayed a higher incidence of encapsulated islet tumors (IT), concomitant with a decrease of widely invasive carcinomas (IC-2). The decreased incidence of IC-2 suggested that loss of COUP-TFII inhibited the invasion of pancreatic β tumor cells.

It has been shown that angiogenic islets are formed at 5 to 10 weeks of age and play a crucial role during β-cell...
tumorigenesis. We hypothesized that the inhibited tumor growth caused by the loss of COUP-TFII might be due to a defect in the angiogenic switch. To this end, we isolated angiogenic islets from 10.5-week-old control and mutant mice to address a possible role for COUP-TFII in the angiogenic switch. We observed that COUP-TFII–deficient mice exhibited a 60% decrease in the number of reddish angiogenic islets compared with control animals (Fig. 1D). The angiogenic switch defects exhibited by COUP-TFII–mutant mice indicate that COUP-TFII is critically involved in the regulation of the balance between pro- and antiangiogenic factors during pancreatic tumorigenesis.

Loss of COUP-TFII impairs tumor angiogenesis

Because loss of COUP-TFII resulted in a significant reduction of angiogenic islet formation, we hypothesized that COUP-TFII might be important for tumor angiogenesis. Morphologic analysis of lesions from control mice showed hypervascularized “red islets” with a microhemorrhaging vasculature, indicative of a robust angiogenic response in control mice (Fig. 2A, top). In sharp contrast, the islets from COUP-TFII–deficient mice displayed a decrease in the vasculature network, suggesting that angiogenesis was compromised in the absence of COUP-TFII. Furthermore, histologic analysis revealed that there was microhemorrhaging, as observed by the accumulation of erythrocytes inside the islets in control mice (Fig. 2A, bottom), whereas this was largely absent in COUP-TFII–mutant mice. To ensure that the difference observed was due to an angiogenic defect in mutant mice, we performed CD31 and NG2 immunostaining to examine vessel density within the angiogenic islets. It is clear that the vascular network...
was well formed in the control angiogenic islets, but not in COUP-TFII–mutant mice (Fig. 2B). Quantification of the vascular network showed that there was about 50% reduction in vessel density and the number of pericytes in the tumors from the mutant mice. Collectively, these results support the notion that COUP-TFII is important for pathologic neoangiogenesis.

The angiogenic defects observed in the tumor regions from the mutant mice suggested that tumor cells might be deprived of nutrients and oxygen and would exit the cell cycle and undergo apoptosis. In agreement with this notion, we found that there was a substantial reduction of proliferation and survival of tumor cells as evaluated by phosphorylated histone H3 and cleaved caspase-3 immunostaining, respectively; the results are quantified in Fig. 2C. Taken together, these data suggest that the loss of COUP-TFII results in angiogenesis defects and subsequent reduction of tumor growth.

**Inactivation of COUP-TFII inhibits tumor metastasis and lymphangiogenesis**

Because angiogenesis and lymphangiogenesis are necessary for the growth and metastatic spread of solid tumors, we asked whether metastasis was also inhibited by the loss of COUP-TFII. In the RIP-Tag2 model, β tumor cells would follow routes of natural drainage and reach the marginal sinus of pancreatic mesenteric lymph node, thus forming metastases at these sites (3). We therefore analyzed regional lymph nodes in control and COUP-TFII–mutant mice at 14 weeks of age. Immunohistochemical staining of insulin expression in mesenteric lymph nodes showed that metastatic tumor cells were indeed observed in about 28% of control mice (6 of 21; Fig. 3A). In contrast, metastasis was not observed in COUP-TFII–mutant mice (0 of 21). In accordance, quantitative reverse transcription-PCR (RT-PCR) analysis showed that transcripts of T antigen and Pdx1 mRNA, which are expressed specifically in the pancreatic β tumor cells,
were indeed detectable in insulin-positive lymph nodes from control mice but were largely absent in COUP-TFII-deficient mice (Fig. 3A, right). Collectively, our results show that the loss of COUP-TFII inhibits tumor metastasis to regional lymph nodes.

The inhibition of lymph node metastasis due to loss of COUP-TFII may arise directly from the effect of COUP-TFII on the lymphatic system, such as decreased lymphangiogenesis, or be indirectly affected by the invasive capacity of the tumor cell itself. Having recently shown that COUP-TFII modulates lymphatic vessel development by regulating the expression of neuropilin-2 (22), we asked whether tumor lymphangiogenesis was also compromised in the absence of COUP-TFII. To this end, the abundance and distribution of lymphatic vessels in the vicinity of control or mutant islet tumors were examined by staining for the lymphatic marker LYVE-1. We found that LYVE-1–expressing lymphatic vessels were distributed largely around but also within the islet tumors in control mice (Fig. 3B). In contrast, the density of lymphatic vessels surrounding the islet tumors was markedly decreased in the absence of COUP-TFII. Furthermore, lymphatic vessels were never observed inside the islet tumors of the mutant mice. Quantification results showed a significant decrease in the percentage of the LYVE-1 covering areas per islet tumor of the mutant mice (Fig. 3C). In the analysis of sections stained for LYVE-1 antibody, we found a significant decrease in the number of disseminated tumor cell emboli within mutant lymphatic vessels. There was around 3.2% lymphatic vessels filled with disseminated tumor cells in the control mice, but the incidence was greatly reduced in the mutant mice (Fig. 3D). This result is consistent with the earlier observation that the tumors from COUP-TFII–mutant mice seemed to be less invasive and less likely to metastasize to other sites. Taken together, we conclude that the loss of COUP-TFII inhibits lymphangiogenesis and tumor metastasis.

**Depletion of COUP-TFII impairs endothelial sprouting**

We previously showed that COUP-TFII regulated a pericyte-derived paracrine signal (Ang1/Tie2 signaling) for the endothelium (15). However, the profound angiogenic defect displayed by COUP-TFII–mutant mice in the RIP-Tag2 model is unlikely only due to the regulation of Ang1 expression by COUP-TFII. Because COUP-TFII was also strongly expressed in endothelial cells, we hypothesized that COUP-TFII might have an intrinsic function in endothelial cells to regulate tumor angiogenesis. To answer this question, we used a three-dimensional spheroidal sprouting assay to examine a possible autonomous role of COUP-TFII in endothelial sprouting. We knocked down COUP-TFII in human primary endothelial cells (HUVECs), and COUP-TFII was efficiently depleted as shown in Fig. 4A. Outgrowth and branching of capillary-like structures, also known as endothelial sprouting, were assessed. We observed that depletion of COUP-TFII abolished endothelial sprouting (Fig. 4A). Endothelial sprouting as measured by the cumulative spraying length was substantially decreased in COUP-TFII knockdown cells (14.5 ± 5.7 μm) in comparison with control cells (924.5 ± 77.7 μm). Similarly, the number of sprouts per spheroid was also reduced in COUP-TFII knockdown cells (0.78 ± 0.22) versus controls (11.0 ± 0.58). These results indicate that COUP-TFII plays an autonomous role in blood endothelial cells to control vessel sprouting.

Endothelial sprouting is a multistep process, involving both proliferation and migration. Indeed, we observed a decrease in the number of proliferating cells subsequent to COUP-TFII depletion over a 4-day period (Fig. 4B). Furthermore, [3H]thymidine incorporation assays revealed that depletion of COUP-TFII resulted in a defect in cell proliferation (Supplementary Fig. S2A). Moreover, flow cytometry analysis indicated that cells with reduced COUP-TFII expression showed a defect in G1–S phase transition (control: 66.3% cells in G\textsubscript{1}, 25.6% cells in S, versus mutant: 78.3% cells in G\textsubscript{1}, 12.6% cells in S; Fig. 4C). Taken together, these results indicate that COUP-TFII is essential for the proliferation of blood endothelial cells.

We next used a wound healing assay to address whether COUP-TFII affects endothelial migration. We found that migration of endothelial cells was impaired when COUP-TFII was knocked down in comparison with the control (Supplementary Fig. S2B). Furthermore, the migration rate of COUP-TFII knockdown cells in response to VEGF-A decreased by about 50% in a chamber migration assay (Fig. 4D). Taken together, these results showed that COUP-TFII promoted vessel sprouting through regulation of blood endothelial cell proliferation and migration.

**Upregulation of VEGFR-1 in islet tumors of COUP-TFII–mutant mice**

To identify the COUP-TFII downstream targets that affected angiogenesis, we isolated angiogenic islet tumors from control and COUP-TFII–mutant mice and performed a candidate gene profiling analysis to assess possible changes in a set of proangiogenic factors. These include VEGF-A, FGF, PDGF, VEGFR-2, VEGFR-1, sVEGFR-1, PDGFR-1, FGFR-1, and members of the angiopoietin family (Fig. 5A). We found that the expression levels of VEGFR-1 and sVEGFR-1 were significantly increased in the absence of COUP-TFII, which was further confirmed by Western blot analysis (Fig. 5B). Notably, the increased expression of VEGF-A in the mutant mice was very likely due to the secondary effects of the hypoxia condition and angiogenic defects in COUP-TFII–deficient mice. It is well established that VEGFR-1 is expressed in blood endothelial cells and counteracts the positive mitogenic signal of VEGFR-2 by sequestering the VEGF ligand (23, 24). For this reason, we sought to examine whether VEGF/VEGFR-2 signaling, the major angiogenic signaling for vascular formation, was impaired in the tumors of COUP-TFII–mutant mice. Our results revealed that the loss of COUP-TFII indeed compromised VEGFR-2 signaling, as indicated by the reduced phosphorylated level of VEGFR-2 in the mutant angiogenic islets (Fig. 5C). Collectively, COUP-TFII might modulate VEGFR-2 signaling through regulation of VEGFR-1 expression.
COUP-TFII modulates VEGF/VEGFR-2 signaling through direct regulation of VEGFR-1 expression

To elucidate the underlying molecular mechanism on how COUP-TFII regulated VEGFR-1 expression within endothelial cells, we knocked down COUP-TFII in HUVECs and examined whether the expression levels of VEGFR-1 were consistently increased in the COUP-TFII-depleted cells. By quantitative RT-PCR and immunoblotting analysis, we showed that ablation of COUP-TFII increased VEGFR-1 levels by nearly 3-fold (Supplementary Fig. S3; Fig. 6A). A similar increase was also observed in the levels of sVEGFR-1 (Supplementary Fig. S3), which primarily acts as a decoy receptor to sequester VEGF-A.

Because VEGFR-1 expression was increased, we asked whether VEGFR-2 signaling is perturbed. We found that the phosphorylated level of VEGFR-2 decreased concomitantly with an increase in VEGFR-1 expression, whereas the total levels of VEGFR-2 remained the same in control and COUP-TFII knockdown cells (Fig. 6A). Thus, our results supported the in vivo observation that loss of COUP-TFII damped VEGF/VEGFR-2 signaling. Because we hypothesized that the upregulation of VEGFR-1 was a major reason for the decreased VEGFR-2 signaling in the absence of COUP-TFII, it is anticipated that reduced VEGFR-1 expression level in COUP-TFII-depleted cells will restore VEGFR-2 signaling. To this end, we treated COUP-TFII knockdown cells with VEGFR-1 antibody to neutralize functional VEGFR-1. As expected, we found that the phosphorylated level of VEGFR-2 was largely restored in COUP-TFII-depleted cells treated with VEGFR-1 antibody, whereas there was no discernable effect in control cells (Fig. 6B), suggesting that the ablation of COUP-TFII impaired VEGF/VEGFR-2 signaling mainly by increasing VEGFR-1 expression.

Previous studies have shown that COUP-TFII can directly bind the conserved DR binding site to repress target gene expression (9). To address whether COUP-TFII directly regulates VEGFR-1 expression, we used a chromatin immuno-precipitation assay to examine if COUP-TFII could be recruited to the VEGFR-1 promoter via conserved COUP-TFII response elements. Figure 6C shows that COUP-TFII...
was preferentially recruited to the region containing a conserved DR2 COUP-TFII response element in comparison with nonspecific IgG, whereas there was no difference in COUP-TFII recruitment to an intragenic region lacking a COUP-TFII binding site. Thus, COUP-TFII directly binds to the VEGFR-1 promoter through a conserved DR2 binding site to negatively regulate VEGFR-1 expression.

Finally, if the sprouting defects were largely contributed by the elevated expression of VEGFR-1, we would anticipate that an inhibition of VEGFR-1 expression might rescue the sprouting defects exhibited by COUP-TFII knockdown cells. To address this, we knocked down VEGFR-1 expression in COUP-TFII–deficient cells and found that endothelial sprouting length and the number of sprouts were partially restored (Fig. 6D). Collectively, our results suggest that COUP-TFII regulates VEGFR-2 signaling by directly repressing VEGFR-1 expression.

Discussion

In the present study, we took advantage of a well-defined β-cell carcinogenesis model to assess the role of COUP-TFII during the angiogenic switch as well as pancreatic tumor progression. Our results highlight the notion that COUP-TFII is critical for the angiogenic switch during tumorigenesis. Notably, COUP-TFII is a member of the nuclear receptor superfamily whose activity can be regulated by small diffusible ligands. Indeed, structural analysis has shown that COUP-TFII contains a potential ligand binding pocket, and its activity could be stimulated by retinoids (25), thus raising the interesting possibility that antagonists for COUP-TFII can be found and supporting COUP-TFII as a novel “druggable” target for the treatment of tumors.

Using a xenograft model, we previously showed that COUP-TFII directly regulates Ang1 expression in pericytes to modulate angiogenesis (15). However, angiogenic profile analysis indicated that there was little change between control and mutant animals with respect to Ang1 expression (Fig. 5). This is not unexpected because Casanovas and colleagues showed that Ang1 was induced by the islet tumor cells in this cancer model (26), and the increase of Ang1 in the tumor cells may mask the downregulation of COUP-TFII in the tumor environment. To assess if COUP-TFII regulates Ang1 expression in the tumor microenvironment in the current model, we isolated adjacent normal pancreatic tissue and pancreatic islet tumor and compared the Ang1 levels in control and mutant mice. Our results indicated that Ang1 expression was indeed decreased in the pancreatic tissue adjacent to the tumor in COUP-TFII–mutant mice (Supplementary Fig. S4). These results are consistent with our previous findings (15). As expected, the expression of Ang1 in islet tumors remains the same in control and mutant tumors owing to the high expression of Ang1 elicited by β tumor cells.

Here, we provide compelling evidence that COUP-TFII controls VEGFR-2 signaling by directly suppressing the endothelial expression of VEGFR-1 and sVEGFR-1 (27). Consistent with the results from the pancreatic islet tumor model, we found that VEGFR-1 expression is consistently upregulated in the Matrigel Plug model (Supplementary Fig. S5), indicating that regulation of VEGFR-1 expression by COUP-TFII is a general mechanism in both physiologic and pathologic neoangiogenesis processes. In the RIP-Tag2 tumors, the results from genetic inactivation of VEGF alleles in pancreatic β cells (28, 29) have documented that VEGFR-2 signaling is a key driver of vascular growth. This notion is supported by the observation that sVEGF-R1 delivered by an adenovirus vector
is a potent inhibitor of RIP-Tag2 tumor angiogenesis and tumor progression (30). Although it has been reported that VEGFR-1 is also expressed in many nonendothelial cell types, including macrophages and various tumor cells (31), in situ hybridization analysis revealed that VEGFR-1 expression is detectable only in the islet vasculature, at least in this tumor model (32–34). Therefore, we conclude that the in vivo upregulation of VEGFR-1 expression in COUP-TFI1–mutant mice is primarily attributed to the endothelial regulation by COUP-TFI1.

Our results suggest that COUP-TFI1 is essential for tumor-induced neo-lymphangiogenesis. Intriguingly, we found that COUP-TFI1 did not regulate the transcription of VEGFR-1 in lymphatic endothelial cells (Supplementary Fig. S3). Therefore, lymphangiogenesis defects displayed by COUP-TFI1–mutant mice are unlikely due to VEGFR-1 regulation by COUP-TFI1. However, we recently showed that COUP-TFI1 enhanced the pro-lymphangiogenic actions of VEGF-C by directly stimulating the expression of neuropilin-2, a coreceptor for VEGF-C, to control lymphatic vessel sprouting (22). Loss of COUP-TFI1 significantly compromised VEGFR-3 signaling, which is a major receptor mediating VEGF signaling for lymphangiogenesis. Furthermore, COUP-TFI1 has been shown to physically and functionally interact with Prox1, a key regulator of lymphatic endothelial cells (19, 35, 36), raising the interesting possibility that COUP-TFI1 acts jointly with Prox1 to regulate tumor lymphangiogenesis. In this regard, COUP-TFI1 might exert its effect in tumor lymphangiogenesis through regulation of VEGFR-3/Nrp2 signaling and interactions with Prox1.

In summary, our results reveal a central role for COUP-TFI1 in the pathologic angiogenic and lymphangiogenic responses and support COUP-TFI1 as a promising new target for antiangiogenic therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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