Pigment Epithelium-derived Factor (PEDF) Blocks Wnt3a Protein-induced Autophagy in Pancreatic Intraepithelial Neoplasms*

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An increase in autophagy characterizes pancreatic carcinogenesis, but the signals that regulate this process are incompletely understood. Because canonical Wnt/β-catenin signaling is necessary for the transition from early to advanced pancreatic intraepithelial neoplasia (PanIN) lesions, we assessed whether Wnt ligands and endogenous inhibitors of Wnt signaling modulate autophagy. In this study, canonical Wnt3a ligand induced autophagy markers and vacuoles in murine PanIN cells. Furthermore, pigment epithelium-derived factor (PEDF), a secreted glycoprotein known for its anti-tumor properties, blocked Wnt3a-directed induction of autophagy proteins. Autophagy inhibition was complemented by reciprocal regulation of the oxidative stress enzymes, superoxide dismutase 2 (SOD2) and catalase. Transcriptional control of Sod2 expression was mediated by PEDF-induced NFκB nuclear translocation. PEDF-dependent SOD2 expression in PanIN lesions was recapitulated in a murine model of PanIN formation where PEDF was deleted. In human PanIN lesions, co-expression of PEDF and SOD2 was observed in the majority of early PanIN lesions (47/50, 94%), whereas PEDF and SOD2 immunolocalization in high-grade human PanIN-2/3 was uncommon (7/50, 14%). These results indicate that PEDF regulates autophagy through coordinate Wnt signaling blockade and NFκB activation.

Pancreatic ductal adenocarcinoma (PDAC) is projected to become the second leading cause of cancer-related deaths in the United States by 2030 (1). Improvements in progression-free survival have occurred, but the overall survival for PDAC patients remains dismal (2). Targeting precursors of PDAC, pancreatic intraepithelial neoplasia (PanIN) lesions, has been proposed as a way of identifying high-risk patients and preventing PDAC development. PanINs, however, are commonly found in autopsy series of non-malignant pancreatic disease. Therefore, insights into novel factors that determine PanIN biology may reveal regulators of indolent versus aggressive disease.

Autophagy is an essential regulator of pancreatic carcinogenesis (3, 4). This conserved cellular recycling mechanism occurs at basal levels in the normal pancreatic ductal epithelium and early PanIN-1 lesions and increases in PanIN-2/3 and invasive PDAC to promote cancer cell survival (5, 6). Murine models of mutant Kras recapitulate pancreatic carcinogenesis where autophagy increases from PanINs through PDAC (6, 7). Functionally, autophagy maintains cellular homeostasis by removing damaged organelles that arise from heightened levels of reactive oxygen species (ROS) (8). Elevated ROS therefore stimulate autophagy, but the endogenous factors that curb ROS production and autophagy induction are incompletely understood (9).

Activation of developmental signaling pathways necessary for pancreatic carcinogenesis can regulate autophagy. For instance, Wnt/β-catenin signaling is necessary for PanIN development and activates mitochondrial biogenesis, thereby increasing ROS formation and autophagy (10, 11). Mice where β-catenin is genetically deleted or its inhibitors overexpressed in the context of mutant KrasG12D form early PanIN lesions that do not progress (10). These studies implicate Wnt/β-catenin signaling, ROS generation, and autophagy induction in PanIN development. Thus, blockade of Wnt signaling may reduce ROS levels and ameliorate autophagy, thereby decreasing cancer cell survival.

To test the hypothesis that inhibitors of Wnt/β-catenin can reduce autophagy and ROS formation, we examined the role of pigment epithelium-derived factor (PEDF) in PanIN biology. PEDF is a 50-kDa non-inhibitory SERPIN with broad anti-tumor properties (12). In PDAC, retained tumor PEDF expression is associated with improved patient survival, and its experimental delivery inhibits PDAC growth in vivo (13, 14). These effects have typically been attributed to an anti-angiogenic effect (12, 15). The surprising discovery of the PEDF null state in humans as the cause of a classic genetic bone disease, osteogenesis imperfecta type VI, led to investigation of its role in developmental signaling pathways such as Wnt/β-catenin signaling, a major effector for bone development (16–19). Our

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2 The abbreviations used are: PDAC, pancreatic ductal adenocarcinoma; PEDF, pigment epithelium-derived factor; PanIN, pancreatic intraepithelial neoplasia; SOD2, superoxide dismutase 2; ROS, reactive oxygen species; HCQ, hydroxychloroquine; IF, immunofluorescence.
group and others have applied this insight from the PEDF null state in humans to identify a Wnt inhibitory function for PEDF in epithelial and cancer cells (20–22).

Here, we investigated whether PEDF inhibits Wnt/β-catenin signaling in murine PanIN cells and determined its effects on autophagy. Our results indicate that canonical Wnt3a ligand activates autophagy in PanIN cells, and autophagy in turn inhibits Wnt activation through inhibition of low density lipoprotein receptor-related protein 6 (LRP6). PEDF inhibits canonical Wnt/β-catenin signaling, Wnt3a-induced autophagy, and elevates superoxide dismutase (SOD) 2 through NFκB activation. In mice, SOD2 expression was distinctly lower in the KrasG12D/PEDF KO double mutant compared with the KrasG12D mutant alone. Human PanIN lesions demonstrated SOD2 and PEDF localization in simple columnar cells (PanIN-1), whereas loss of expression occurred in cells with advanced (PanIN 2/3) histological features. Our findings highlight a mechanistic role for PEDF’s anti-tumor properties through the blockade of Wnt/β-catenin-induced autophagy and the activation of NFκB-dependent responses to modulate ROS in PanIN cells.

Results

Canonical Wnt Regulates Autophagy in Murine PanIN Cells—Autophagy increases as PanIN lesions progress. We tested protein levels of LC3 and autophagic vacuole formation in two murine PanIN cell lines (PI5505, PanIN-1/2; PI34, PanIN-3) (Fig. 1, A and B). Compared with PI5505 cells, protein levels of LC3 and autophagic vacuoles were significantly increased in PI34 (Fig. 1, A and B). The mCherry-YFP-LC3 construct results in a distinct mCherry and YFP labeling pattern (23). In the acidic conditions found in lysosomes, YFP loses fluorescence while mCherry fluorescence remains. Using this construct, PI34 cells demonstrated higher YFP and mCherry...
levels than PI5505 cells, consistent with the levels of LC3 and autophagic vacuole formation (Fig. 1C). Thus, murine PanIN cells derived from the Kras mutant mouse model reveal a progressive increase in autophagy with increasing histological grade (7).

To investigate whether canonical Wnt ligands regulate autophagy, protein levels of LC3 were determined 6 h post-Wnt3a treatment (100 ng/ml). Wnt3a increased LC3-II levels and decreased p62 content (Fig. 1B), a marker of enhanced autophagic flux. Enhanced mCherry fluorescence also occurred in response to Wnt3a treatment (Fig. 1C). Autophagic vacuole formation increased in PanIN cells in a time-dependent manner after Wnt3a (Fig. 1D). Chemical manipulation of canonical Wnt signaling further demonstrated Wnt-dependent effects on autophagic vacuole formation. Increasing concentrations of CHIR99021, a Wnt activator, increased autophagic vacuole formation and LC3-II levels (Fig. 1, E and F). In contrast, IWP2, a Wnt inhibitor, decreased autophagic vacuole formation and LC3-II protein levels (Fig. 1, G and H). These findings indicate that canonical Wnt signaling regulates autophagy in PanIN cells.

**Autophagy Induction Is Inversely Related to LRP6 Activation**—Autophagy can be induced by starvation conditions (24). Low (1 mM) glucose induced LC3-II and autophagic vacuole formation in PI5505 and PI34 cells and reduced p-LRP6 and LRP6 without changing β-catenin activation (Fig. 2, A–D). This effect was confirmed using rapamycin, an autophagy

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**FIGURE 2. Activation of autophagy inhibits activation of the Wnt receptor LRP6.** A, expression of mCherry-YFP-LC3 was determined in response to different concentrations of glucose. B, autophagic vacuole formation under different concentrations of glucose treatment in PanIN cells was determined. C, protein levels of p-LRP6, LRP6, active β-catenin, β-catenin, and LC3 were determined by immunoblotting in response to different concentrations of glucose. D, quantification of p-LRP6/β-actin. E, levels of active LRP6 and Wnt signaling components after exposure to rapamycin, an autophagy inducer; and F, HCQ, an autophagy inhibitor. G, quantification of protein levels obtained in F. Data presented as mean ± S.D. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
**TABLE 1**

Mouse MnSOD (SOD2) promoter sequence

DNA sequence of mouse SOD2 promoter is shown. The predicted binding sites (red), primers for binding sites NfκB1 (black, underlined), NfκB2 (black, underlined), and NfκB3 (blue, underlined) are shown. ATG (bold italics) is the translation start site.

| Model ID | Model name | Score | Relative score | Start | End | Strand | Predicted site sequence |
|----------|------------|-------|----------------|-------|------|--------|-------------------------|
| MA0105.1 | NfκB1      | 7.873 | 0.8262201960139 | 280   | 289  | −1     | GGGGAATGGC              |
| MA0105.1 | NfκB1      | 9.869 | 0.874557955765429 | 800   | 809  | −1     | GAGACTTTCC              |
| MA0105.1 | NfκB1      | 7.701 | 0.8202054952156393 | 811   | 820  | 1      | AGGGTTTTCC              |
| MA0105.1 | NfκB1      | 9.006 | 0.853658666782681 | 918   | 927  | −1     | GGGGAACCCCG              |
| MA0105.1 | NfκB1      | 9.096 | 0.855838019535528 | 918   | 927  | −1     | GGGGAACCCCG              |

**TABLE 2**

Predicted NfκB binding to promoter sites of the mouse SOD2 gene

Five putative NfκB sites were predicted with these settings (80%) in sequence named SOD2. This analysis has high sensitivity but low selectivity. In other words, while functional activity will be detected in most cases, most predictions will correspond to sites bound in vitro but with no function in vivo. A number of additional constraints of the analysis can improve the prediction; phylogenetic footprinting is the most common.

We recommend using the ConSite service, which uses the JASPAR database. The review in Ref. 47 gives a comprehensive overview of transcription binding site prediction. PEDF binding to putative sites of the mouse SOD2 promoter by JASPAR database.

inducer through inhibition of mechanistic target of rapamycin signaling (Fig. 2F). Hydroxychloroquine (HCQ), an autophagy inhibitor, increased p-LRP6 levels in PanIN cells (Fig. 2, F and G). Thus, autophagic activity negatively regulates components of the Wnt signaling apparatus such as LRP6, the co-receptor for canonical Wnt signaling. This suggests an internal negative feedback loop to curb Wnt signaling in conditions of nutrient deprivation.

**PEDF Blocks Canonical Wnt Signaling**—PEDF inhibits canonical Wnt signaling in multiple cell types (20–22). To address whether PEDF could function as a Wnt antagonist in PanINs, PEDF-mediated blockade of Wnt3a was assessed. Wnt3a increased active β-catenin in both PI5505 and PI34 cells, and PEDF inhibited this effect (Fig. 3A). Inhibitory effects were also detected in cellular fractionation extracts isolated from PanIN cells (Fig. 3A). Immunofluorescence imaging demonstrated that PEDF inhibits Wnt3a-mediated β-catenin translocation (arrows) in both PanIN cell lines (Fig. 3B). T-cell transcription factor 4 (TCF4) is the downstream effector of β-catenin responses through a transcriptional complex. PEDF protein inhibited activity of the TCF4-luciferase reporter (Fig. 3C) in the basal state and after Wnt3a exposure (Fig. 3C). Either PEDF gene expression or adding the conditioned medium from PEDF-transfected cells yielded similar results in both PanIN cell lines (Fig. 3D). CyclinD1 and c-Jun are downstream targets of TCF4/β-catenin and reflect Wnt signaling activation (25). CyclinD1 and c-Jun expression were significantly inhibited by PEDF (Fig. 3, E and F), indicating that canonical Wnt signaling is blocked by PEDF in PanIN cells.

**PEDF Inhibits Wnt3a-mediated Autophagy**—Because canonical Wnt ligand enhances autophagy and PEDF blocks Wnt signaling, we next determined whether PEDF could inhibit autophagic responses. PEDF reduced LC3-II (Fig. 4A) and autophagic vacuole formation (Fig. 4B) in PI5505 and PI34 cells under basal conditions. PEDF also inhibited Wnt3a-induced LC3-II protein and autophagic vacuole formation in PI5505 and PI34 cells (Fig. 4, C and D). Analogous results were observed in PanIN cells transfected with mCherry-YFP-LC3 (data not shown). PEDF similarly blocked autophagic vacuole formation in rapamycin-treated cells (Fig. 4E). PEDF also inhibited hydroxychloroquine-mediated LC3-II up-regulation, indicating that PEDF-mediated LC3 inhibition occurs at an earlier stage of autophagy during the formation of autophagic vacuoles (Fig. 4F).

**PEDF Suppresses Catatase and Increases Superoxide Dismutase in PanIN Cells**—ROS levels stimulate autophagy, and PEDF has been previously identified as an endogenous factor to counter ROS in multiple cell-based systems (8, 26). We examined PEDF-dependent effects on ROS levels and autophagy in PanIN cells. Under baseline conditions, advanced PanIN cells (PI34) had higher H2O2 levels than low grade PanIN cells (PI5505) (data not shown). Exogenous H2O2 induced autophagy by up-regulating LC3-II and autophagic vacuole formation in both PanIN cells (data not shown). We also tested whether PEDF modulates H2O2 generation in PanIN cells, and we observed a small but statistically significant (~10%) induction of H2O2 in response to PEDF treatment (Fig. 4G). Blockade of autophagy with HCQ similarly led to an increase in H2O2
The Wnt inhibitor IWP2 also increased H$_2$O$_2$ by more than 50% in the PI34 cells (Fig. 4I). These results indicate that Wnt blockade enhances H$_2$O$_2$ levels in murine PanIN cells. Because we had anticipated an overall reduction in ROS with Wnt blockade and PEDF treatment, but instead found enhanced H$_2$O$_2$ levels, we examined whether PEDF had selective effects on antioxidant enzymes involved in H$_2$O$_2$ metabolism as follows: SOD2, a mitochondrial matrix protein that converts the superoxide anion (O$_2^-$) to H$_2$O$_2$, and catalase that converts H$_2$O$_2$ to water. In fact, PEDF increased SOD2 protein levels but decreased catalase in both PanIN cell lines (Fig. 4J). Thus, the increase in H$_2$O$_2$ seen with PEDF exposure parallels the consistent decrease in catalase levels seen with PEDF exposure. In addition, PEDF significantly increased mRNA expression of SOD2 in both PanIN cells (Fig. 4K), indicating that PEDF transcriptionally regulates SOD2.

PEDF Regulates SOD2 Expression through NFκB Activation—We next evaluated how PEDF alters Sod2 transcription. NFκB increases Sod2 expression, and previous studies identified that PEDF provides protective effects against oxidative injury by inducing NFκB activity (27, 28). To test PEDF-mediated regulation of SOD2, NFκB translocation and nuclear activity on the Sod2 promoter was assessed. PEDF led to p65 translocation into the nucleus (Fig. 5, A and D) and enhanced NFκB-luciferase activity in both PI5505 and PI34 cells (Fig. 5B and C). Chromatin immunoprecipitation (ChIP) studies revealed enhanced transcriptional responses in the presence of PEDF compared with control-treated cells (Fig. 5G). Electrophoretic mobility shift assay (EMSA) was performed using wild type and mutant DNA oligonucleotide-targeting binding sites for NFκB on the murine Sod2 promoter with or without biotin labeling (Table 4). Adding PEDF increased the binding affinity of NFκB to both binding sequences (Fig. 5H). Mutation of these binding sites led to loss of greater than 80% binding activity for the NFκB site and
PEDF Regulates SOD2 and Autophagy in PanINs

~50% binding activity for the NFκB2 site. Thus, PEDF activates NFκB-mediated transcription of SOD2 through two sites on the Sod2 promoter with preferential binding to the NFκB1 site.

Loss of SOD2 Occurs in Advanced PanINs in Murine and Human Pancreatic Tissues—Increased expression of SOD2 has been reported with advancing grades of cancer, but its expression levels in PanINs remains unclear. We determined whether PEDF regulates SOD2 expression in transgenic p48-Cre;LSL-KrasG12D (herein referred to as KC) and KC/PEDF KO mice. SOD2 staining was barely detectable in KC/PEDF KO mice, although KC mice demonstrated SOD2 labeling in acinar-ductal metaplasia and early PanINs in KC mice (Fig. 6A). Next, human PDAC sections were analyzed and PanIN lesions stained for SOD2. Within the same PanIN lesions containing both low and high grade PanIN cells, SOD2 labeling was diminished in higher grade PanIN cells (arrows, Fig. 6B). In contrast, simple columnar cells (PanIN-1) within the same PanIN lesion displayed both SOD2 and PEDF labeling (arrowheads, Fig. 6B).

Quantification data showed that 47 out 50 PanIN-1 lesions had positive SOD2 labeling, whereas only a minority of PanIN-2/3 displayed SOD2 (Fig. 6C). We evaluated the autophagy adaptor protein p62 in relation to PanIN histological grade and SOD2/PEDF localization. Immunolabeling of SOD2 and PEDF occurred in simple columnar cells (Fig. 6D, arrowheads), although p62 was prominent in advanced but not early PanIN cells (Fig. 6D, arrows). This inverse correlation between p62

FIGURE 4. PEDF inhibits Wnt3a-directed autophagy. A, LC3 levels after PEDF (300 ng/ml) for 2 or 24 h. B, autophagic vacuole formation after PEDF (300 ng/ml) (n = 2, mean ± S.D.), C, PanIN cells were pretreated with PEDF (300 ng/ml) or untreated for 2 h, followed by Wnt3a (100 ng/ml) treatment or mock treatment for 6 h, and LC3 levels were determined. E and F, PEDF blunts the increase in autophagic vacuole formation induced by Wnt3a (100 ng/ml) (D), rapamycin 1 nM (E), or HCQ 25 μM (F). G and H, H2O2 levels after PEDF (300 ng/ml) (G) and HCQ 24 h of treatment (H). I, normalized H2O2 expression in response to IWP-2 (25 μM). J, SOD2 and catalase levels after PEDF (300 ng/ml) exposure. K, quantification of SOD2/β-actin ratio. Loading control for Fig. 4, A and J are identical due to use of the same membrane to blot for two different targets. Data are presented as mean ± S.D. Significance was calculated using unpaired two-tailed Student’s t test. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
PEDF Regulates SOD2 and Autophagy in PanINs
and PEDF is consistent with PEDF-mediated regulation of SOD2 and autophagy, and is diminished in advanced PanIN lesions.

**Discussion**

Reciprocal regulation of canonical Wnt signaling and NFκB activity couples two fundamental and mutually exclusive transcriptional programs that direct cellular activity toward proliferative versus cellular protective responses (29, 30). In this study we defined a Wnt inhibitory activity for PEDF in PanIN cells that resulted in NFκB activation (21). Thus, PEDF functional activity as a tumor inhibitor stems in part from its ability to inhibit Wnt signaling activation. Therefore, PEDF stimulates SOD2 and blocks catalase, thereby limiting ROS levels. This is consistent with previous studies using constitutively active mutant Kras models, increased ROS advances PanIN cell histology through the induction of growth factor signaling, whereasblunting ROS levels impedes PanIN progression. These results highlight a role for SOD2 in pancreatic carcinogenesis and PEDF's role in its regulation. In the murine Kras model, increased ROS advances PanIN cell histology through the induction of growth factor signaling, whereas blunting ROS levels impedes PanIN progression. Thus, regulation of SOD2 in PanIN cells supports a novel anti-cancer cell mode of action for PEDF.

A central finding of this study is that PEDF can direct NFκB nuclear translocation/activity, enhance NFκB binding to the Sod2 promoter, and induce SOD2 levels. Previous studies in neuronal cells demonstrated that PEDF regulates protective responses through an NFκB-dependent mechanism (27). This study links PEDF-mediated blockade of Wnt/β-catenin signaling with PEDF-directed NFκB activation, which increased measures to curb specific ROS. It has been demonstrated that mutant Kras cells generate excess ROS that induces signaling proteins that favor malignant transformation. In contrast, PEDF stimulated SOD2 and blocked catalase, thereby resulting in an incremental and defined increase in H2O2 levels. Whether this selective increase in ROS species with PEDF exposure maintains protective versus harmful NFκB responses remains unclear (35, 37).

Because PEDF selectively modulates ROS by increasing SOD2/decreasing catalase, this supports the view that PEDF fine-tunes, rather than eliminates, the endogenous levels of ROS. Because a certain degree of ROS is necessary for signal transduction pathways, this suggests that reciprocal reg-

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**TABLE 3**  
ChIP primers for NFκB-binding sites on mouse SOD2 promoter

| Binding sites | Forward | Reverse |
|---------------|---------|---------|
| NFκB1         | AGACACAGCTGACATCCTGTTGCA (sense) | CAGTACAGGGCTAGGTAAGGG (antisense) |
| NFκB2         | GGGCCTTCTTATCCATTAAGT (sense) | CAGTACAGGGCTAGGTAAGGG (antisense) |
| NFκB3         | GTAGTGCCCTTCACTGAG (sense) | CAGTACAGGGCTAGGTAAGGG (antisense) |

**TABLE 4**  
Oligonucleotides used for EMSA experiments

| Binding sites | Forward | Reverse |
|---------------|---------|---------|
| NFκB1         | CTCCTAGCGAGAAGCATCCTGTTGCA | CGGTATGGGCTAGGGTAAGGG |
| NFκB2         | CCAAGAACGAGGCTGCTTCCGGAAGATT | CAGTACAGGGCTAGGTAAGGG |
| Mutant NFκB1  | CTCCTAGCGAGAAGCATCCTGTTGCA | CAGTACAGGGCTAGGTAAGGG |
| Mutant NFκB2  | CCAAGAACGAGGCTGCTTCCGGAAGATT | CAGTACAGGGCTAGGTAAGGG |

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**FIGURE 5. PEDF induces NFκB nuclear translocation and transcriptional activation.** A, determination of cytoplasmic (Cyto) and nuclear fractions for p65 levels in response to PEDF 24 h (300 ng/ml) exposure. GAPDH and lamin serve as the controls for cytoplasmic and nuclear fractions, respectively. B and C, NFκB luciferase reporter activity increases in response to PEDF. D, IF imaging of p65 in response to PEDF (300 ng/ml). E, representation of three putative binding sites for NFκB on the murine Sod2 promoter. Ctrl, control. F, chromatin immunoprecipitation assays of NFκB binding to the murine Sod2 promoter sequences in the presence and absence of 24 h treatment of PEDF (300 ng/ml). (n = 2, triplicate, mean ± S.D.) LIGG was used as negative control. Primer sequences are shown in Table 3. G, amplification curves of ChIP assays. H, EMSA of NFκB binding to the murine Sod2 promoter sequences in the presence and absence of 24-h treatment of PEDF (300 ng/ml). #, Epstein-Barr nuclear antigen control extract. Data were presented as mean ± S.D. Significance was calculated using unpaired two-tailed Student’s t test. ***, p < 0.001. SS, supershift; NE, nuclear extract.
ulation of Wnt and NFκB signaling by PEDF functions to maintain ROS homeostasis. With advancing histological features, loss of PEDF occurs and may contribute to dysregulation of ROS and the enhancement of pro-tumor signaling pathways. Future studies examining whether reciprocal regulation of SOD2 and catalase occurs in other cell types and how this alters signaling pathways will allow assessment of PEDF’s anti-cancer properties within this context.

In summary, Wnt3a promotes autophagy in murine PanIN cells, which can be blocked by PEDF. Mechanistically, PEDF inhibits canonical Wnt signaling and permits NFκB activation to enhance SOD2 expression. In human PanIN lesions, co-localization of PEDF and SOD2 occurs in early PanIN lesions and is lost in advanced PanINs. This was confirmed in genetically engineered mouse models of pancreatic carcinogenesis and PEDF deficiency. These data provide additional evidence to support direct anti-cancer cell properties of PEDF through the inhibition of canonical Wnt/β-catenin signaling.

Experimental Procedures

Study Approval—All experiments involving the use of mice were performed following protocols approved by either the Institutional Animal Care and Use Committee or local IRB at the Northwestern University (P. J. G.). Patient slides were obtained in a de-identified fashion after approval from the human investigations committee at the Veterans Affairs Connecticut Healthcare System.

Cell Lines and Chemicals—Two primary murine PanIN cell lines, P15505 (PanIN-1/2) and P134 (PanIN-3), were derived from Pdx-Cre;LSL-Kras<sup>G12D</sup>;p16<sup>fl/fl</sup>;YFP mice at 6 weeks of age, prior to the advent of histological pancreatic cancer (42, 43). PanIN cell lines were grown in DMEM (Life Technologies, Inc.) supplemented with high glucose, 10% fetal bovine serum, and 100 μg/ml penicillin/streptomycin in a humidified incubator at 37 °C and 5% CO₂. Antibodies against LC-3 (catalog no. 2775S), p62 (catalog no. 5114S), SOD2 (catalog no. 13141S), catalase
cells were transfected with 1 μg of luciferase reporter and 10 ng/well Renilla luciferase by Lipofectamine 2000 (Life Technologies, Inc.) in each well. After 48 h of transfection, cells were lysed, and then luciferase and Renilla expressions were determined using Dual-Luciferase® reporter assay (Promega, Madison, WI). Triplicate reactions were performed for each sample, and experiments were repeated three times.

H$_2$O$_2$ Detection—H$_2$O$_2$ levels were determined by Image-iT™ LIVE Green Reactive Oxygen Species detection kit (Life Technologies, Inc.). Indicated cells were incubated with 5-(and-6)-carboxy-2,'7'-dichlorodihydrofluorescein diacetate for 30 min at 37 °C. H$_2$O$_2$ levels were measured by flow cytometry according to the manufacturer’s protocol, and values were reported as mean fluorescence intensity.

Autophagy Detection—Autophagic vacuole formation was detected using Cyto-ID autophagy detection kit according to the manufacturer’s instructions (Enzo Life Science, Farmingdale, NY) and quantified by flow cytometry with values reported as mean fluorescence intensity. All experiments were performed at least twice.

Immunoblotting—Cells were washed with PBS and lysed with the M-Per mammalian protein extraction reagent (Thermo Scientific, Waltham, MA) with protease and phosphatase inhibitors (Roche Applied Science, Basel, Switzerland). Nuclear and cytoplasmic extractions from PanIN cells were isolated using NE-PERTM nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Cell lysates were separated using SDS-polyacrylamide gel (Mini-protein TGX precast gels, Bio-Rad) and probed with corresponding antibodies.

Real Time Quantitative PCR—Total RNA was isolated from PanIN cells (TRlzol reagent; Invitrogen), according to the manufacturer’s instruction. Real time PCR was performed as described previously (45). The primers used are as follows: mouse Sod2 primers: forward primer, 5'-CCATTTTTCTGGA-CAAAACCTGA-3', and reverse primer, 5'-GCCGAGAAGTTGTGCATCTA-3'.

Transfection of Autophagy and NFκB Constructs—The mCherry-YFP-LC3 plasmid was provided by Dr. Thomas Melia (Yale University School of Medicine). 5 × 10⁶ PanIN cells were transfected with 1 μg of DNA by Lipofectamine 3000 (Life Technologies, Inc.) in each well. YFP and mCherry expressions were observed after 24 h of transfection. Cells were treated with P PEDF in serum-free medium. For quantification of fluorescence, images of 10 random fields were visualized. TCF4-luciferase reporter and blank vector pcDNA3.1 were provided by Dr. Carlo Spirli (Yale University School of Medicine). NFκB-luciferase reporter was purchased from Qiagen. 5 × 10⁶ PanIN

FIGURE 7. Schematic diagram of findings. Canonical Wnt3a ligand enhances autophagic vacuole formation in PanIN cells. PEDF inhibits the effects of canonical Wnt3a ligand and blocks autophagosome formation. In contrast, PEDF causes increased SOD2 levels and decreased catalase lead to selective modulation of ROS by PEDF. Purple arrows represent PEDF-dependent effects.
of DNA and proteins. This was followed by proteinase K digestion. Immunoprecipitated DNA was amplified by primer pairs corresponding to three NFκB-binding sites in mouse Sod2 promoter by real time PCR. Primers used are shown in Table 3. Triplicate PCRs were performed for each sample, and the expression data were normalized to respective input values. Data are presented as the mean ± S.D.

Electrophoretic Mobility Shift Assay—EMSA was performed using LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific). Wild type or mutant oligonucleotides were labeled with or without 5' and 3' labeled biotin and designed according to NFκB-binding sites in the murine SOD2 promoter (Table 4). Double strand DNA was annealed before the experiment. Following binding of nuclear extracts (1.6 μg) with DNA, samples were separated in 6% acrylamide TBE gel and transferred to positive charged nylon membranes for final development. An excess of unlabeled DNA was used as a negative control. Epstein-Barr nuclear antigen extract with control DNA was used as a positive control.

Immunohistochemistry/Immunofluorescence Staining—Pancreatic tissue was harvested from p48-Cre;LSL-KrasG12D (KC) and double transgenic (KC/PFED KO) mice (33). Immunohistochemistry and immunofluorescence (IF) labeling were performed as described (44). For immunohistochemistry, tissues were fixed with formalin, embedded in paraffin, sectioned, and stained with H&E. For IF staining, tissues were deparaffinized, rehydrated, and blocked with goat serum for 30 min at room temperature. Sections were incubated with primary antibody (1:200) overnight at 4 °C, followed by respective secondary antibodies conjugated to Alexa Fluor 555 or 488 (1:500; Invitrogen) for 1 h at room temperature. After rinsing in PBS, slides were mounted with ProLong Gold with DAPI (Invitrogen). Twenty IF images were obtained using Zeiss Axiosvert fluorescence microscope for each sample. Tissues stained without antibodies were used as negative controls.

IF labeling was performed on PanIN cells using coverslips. Cells were rinsed with PBS, permeabilized with 100% cold methanol at −20 °C for 20 min, and blocked in 2% bovine serum albumin. Samples were incubated with primary antibody at 37 °C for 1 h and followed by secondary antibody at 37 °C for 1 h. Then they were mounted with ProLong Gold with DAPI. Control slides were incubated in secondary antibody only. Twenty IF images were randomly obtained using Zeiss Axiovert Fluorescence Microscope or CLSM 710 spectral confocal laser scanning microscope for each sample. All experiments were repeated at least twice.

Statistical Analysis—Data were presented as average ± S.D. Statistical significance was determined using GraphPad Prism Version 6 software by analysis of variance for multiple groups or by t-test between two groups, and p values <0.05 were considered significant.

Author Contributions—J. G. and C. C. designed and performed experiments; U. S. performed experiments. J. G., X. Z., P. J. G., and G. B. analyzed the data. J. G. and C. C. wrote the paper. C. C. was responsible for conception and oversaw the project. All authors reviewed the results and approved the final version of the manuscript.

PEDF Regulates SOD2 and Autophagy in PanINs

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