Notch Signaling Activated by Replication Stress–Induced Expression of Midkine Drives Epithelial–Mesenchymal Transition and Chemoresistance in Pancreatic Cancer

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Introduction

Insufficient diagnostic tools and therapeutic options for pancreatic ductal adenocarcinoma (PDAC) still substantiate its ranking as fourth leading cause of cancer-related death (1). The combination of an unusual aggressiveness and early metastatic locoregional as well as distant spread reflects the urgent necessity of new therapeutic options for this deadly disease because its incidence nearly equals mortality. This devastating prognosis is partially because of a frequent occurrence of intrinsic or acquired chemoresistance in PDAC specimens against the nucleoside analogue gemcitabine, which is still the standard for chemotherapeutic treatment of locally advanced and metastatic PDAC (2).

The heparin-binding growth factor Midkine (MK) was first identified as a retinoic acid–inducible gene during embryogenesis two decades ago (3). Since then a plethora of cellular MK functions have been described in normal and transformed tissues. Although MK gene expression is weak or undetectable in adults, the MK reexpression in damaged and inflamed tissues has been discussed (4, 5). In several malignancies, including neuroblastomas, colorectal, and breast carcinomas, protumorigenic effects have been linked to MK reexpression although the mechanisms are not completely understood (6–8).

During embryonal development, the epithelial–mesenchymal transition (EMT) is an indispensable mechanism in which extracellular signals convert epithelial into mesenchymal cells. In adult organisms, EMT takes part in the pathology of certain diseases (9). During pathologic processes such as cancer, EMT has been shown to orchestrate a process by which epithelial cells undergo morphologic changes, that is, a transition from an organized cell layer and polarized organization of the cytoskeleton, which is linked to expression of the epithelial adhesion molecule E-cadherin, β-catenin, and γ-catenin, to mesenchymal cells without an organized cell layer showing enhanced pseudopodia formation and subsequently more motility and invasiveness which is linked to loss of epithelial cell–cell junction, reorganization of cytoskeleton, and upregulation of mesenchymal markers vimentin, fibronectin, and α-smooth-muscle-actin (α-SMA; ref. 10–12). EMT is triggered by a tightly regulated interplay of extracellular signals such as soluble growth factors like epidermal growth factor or transforming growth factor β (13, 14). Numerous receptors such as Notch have been identified playing a significant role in the acquisition of EMT in cancer (15). The activation of Notch regulates expression of target genes playing important roles in embryonic development, cell proliferation, and apoptosis. Recently, the activated Notch receptor-2 (Notch-2) was shown to mediate an EMT phenotype in PDAC cells whereas...
chemotherapy (oxaliplatin)-induced Notch-1 activation was shown to be linked to acquired chemoresistance in colon cancer cells (16). Moreover, Notch-2 was recently shown to interact with MK to regulate cell plasticity and motility in human keratinocytes (17).

Materials and Methods

Cell lines
Tumor tissues for establishment of primary chemoresistant PDAC cell lines (PaCa 5061, 5072, and 5156) were taken from patients who underwent total pancreatectoduodenectomy for advanced PDAC in the Surgery, University Medical Center Hamburg (2009). Written informed consent of patients was obtained prior to surgery. The procedure of cell line establishment from tumor tissue was previously published (18). Primary cells were cultured in TUM medium. L3.6pl (19), BxPC-3, and PANCl-1 cells were cultured in Dulbecco’s modified Eagle’s medium or RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum and 200 IU/mL Pen-Strep at 37°C and 5% CO₂. For development of gemcitabine-resistant L3.6pl cells (L3.6pl-Res), cells were exposed to an initial gemcitabine concentration of 0.05 μmol/L. The surviving population was grown to 80% confluence and passaged 3 times over 14 days to ensure stable viability. Cells were then treated with sequentially increased gemcitabine concentrations (0.1 μmol/L, 0.3 μmol/L, 0.5 μmol/L, 1 μmol/L; 21 days each) up to the clinically relevant concentration of 2 μmol/L for 30 days. L3.6pl-Res cells are approximately 20 times more resistant to gemcitabine than L3.6pl cells. All used cell lines were genotyped by DNA fingerprinting (Identifiler-Kit; Applied Biosystems).

Reagents
Gemcitabine was purchased from Eli Lilly and 5-fluourouracil (5-FU) from Sigma. Recombinant human MK (rh-MK) was purchased from R&D Systems and used at concentrations of 20 and 50 ng/mL, respectively. In rh-MK experiments, cells were deprived of serum for 14 hours and treated for 24 hours with 20 or 50 ng/mL.

Notch-2 signaling
PaCa 5061 and PANCl-1 cells (10⁵) were cultured in 6-well plates. Then, cells were cultured in serum-free media for approximately 14 hours. Subsequently, cells were treated for 24 hours with 20 and 50 ng/mL of rh-MK or left untreated and served as control. rh-MK-induced Notch-2 cleavage was detected by using antibodies recognizing the intracellular domain (Notch-2ICD). Functional receptor activation was detected by using antibodies against the downstream target genes Hes-1 and NF-κB/RelA.

RNA isolation and expression analyses
Total RNA were isolated by using TRIzol (Invitrogen). The dried pellet was cleaned with the RNaseasy MiniElute Kit (Qiagen). RNA concentration was measured on a NanoDrop Spectrophotometer (Peqlab). Gene expression profiles of whole human genome were generated by hybridizing either 5 μg of total RNA from PaCa 5061, 5072, and 5156 or 5 μg human total pancreas RNA (Stratagene and Ambion) serving as healthy controls on GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix). Analyses were carried out according to the 1-cycle cDNA synthesis protocol for GeneChip Expression-Analysis (Affymetrix) and achieved data were analyzed using GCOS 1.4.

Cell viability analyses
To determine drug sensitivity, cells were incubated with different concentrations of gemcitabine and 5-FU. Cell proliferation was determined with CellTiter 96 AQueous Cell Proliferation Assay according to the manufacturer’s protocol (Promega). Values for control cells were considered as 100% viability.

Real-time reverse transcriptase PCR
Real-time reverse transcriptase PCR (RT-PCR) was conducted to quantify gene expression or to verify RNAi-mediated downregulation. Total RNA (1 μg) was reverse transcribed by using the Transcriptor cDNA Kit (Roche). PCRs were carried out in a Mastercycler ep-realplex (Eppendorf). Data were analyzed according to the comparative Cₜ method and normalized for cyclophilin expression in each sample.

Plasmids
Cells were transfected with siRNAs against MK and Notch-2 or control siRNA (Santa Cruz) using Lipofectamine (Invitrogen). Notch-2 open reading frame encoding the N-terminal extracellular domain (1-351) was amplified by PCR and sub cloned into p3xFLAG-CMV-14. MK was amplified by PCR and sub cloned for expression into pCS2 (20).

Immunoprecipitation and Western blotting
PANC-1 cells were transfected with indicated plasmids. Following transfection, cells were lysed in RIPA buffer (Sigma) containing ×1 protease inhibitor cocktail (Roche). Supernatants were resolved by SDS-PAGE followed by immunoblotting or immunoprecipitation (IP) as previously described (20). Western blots, IPs, and immunostainings were carried out by using antibodies listed in Supplementary Table S2.

Immunohistochemistry
Stainings were carried out with peroxidase method according to manufacturer’s protocol (R&D Systems). Paraffin-embedded human PDAC specimens were obtained from the Tissue-Bank of the University of Hamburg. PDAC slides (5 μm) were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was done by microwave heating. Endogenous peroxidase activity was blocked by H₂O₂ [1% (v/v) in methanol]. Sections were counterstained with Meyer’s hemalaun-solution (Merck) and permanently mounted. For negative control stainings, normal tissue was stained or appropriate control IgG stainings were carried out.

Data analyses
Expression analyses and achieved data were analyzed by using GCOS 1.4 and scaled to a default target signal value of 150. Absolute and comparative analyses were conducted by...
using MAS 5.0 algorithm. Annotations were analyzed with interactive query analysis. Experiments presented are representative of 3 different repetitions. Data are presented as the mean values ± SE.

Results

**MK is frequently overexpressed in chemoresistant PDAC**

The PDAC cell line PaCa 5061 has recently been characterized in depth *in vitro* and *in vivo* (18). To identify novel players involved in intrinsic or acquired chemoresistance in PDAC, we analyzed the gene expression profile of 3 newly established chemoresistant cell lines (PaCa 5061, 5072, and 5156) in comparison with RNA expression patterns obtained from 2 independent normal pancreas specimens. The native intrinsic gemcitabine resistance was examined in all lines prior to the analysis. To identify chemoresistance-relevant molecules, we searched for "hits" overrepresented more than 5-fold in all resistant lines compared with normal pancreas. Among the top 5 hits, we identified MK as overexpressed in cell lines (Fig. 1A, left). The *P* values and fold changes of selected genes are shown in Table 1 (Supplementary Table S1). To further validate our microarray results, we retested MK overexpression and carried out real-time RT-PCR to quantify MK mRNA in the investigated lines (Fig. 1A, right). We expanded these analyses and quantified MK mRNA expression in more than 30 additional PDAC tissues by real-time RT-PCR (Fig. 1B). We observed a statistically significant mRNA overexpression in more than 50% of the investigated samples (*P* < 0.05). To verify that the MK mRNA expression resulted in robust protein expression, we carried out immunostainings from same tissues or investigated MK protein expression in cell lines and showed that mRNA overexpression resulted in robust protein expression. Consistent with previous observations where MK mRNA was shown to be overexpressed in PDAC (21), we further showed that the mRNA overexpression resulted in robust protein expression (Fig. 1C). Moreover, steady-state MK protein levels were upregulated in chemoresistant PDAC lines and almost undetectable in sensitive cells (Fig. 1D).

Figure 1. MK is frequently overexpressed in chemoresistant PDAC. A, differential MK gene expression in primary chemoresistant PDAC cell lines compared with 2 independent normal pancreas probes (control 1 + 2) obtained by microarray analyses. Left, MK is significantly overrepresented in chemoresistant PDAC lines more than 5-fold (relative units) compared with normal pancreas (*P* < 0.004). Right, real-time RT-PCR was used to quantify MK mRNA expression in chemoresistant lines compared with normal pancreas. **, *P* < 0.01; *, *P* < 0.05. B, real-time RT-PCR–based quantification of MK mRNA expression in PDAC patient samples (n = 31) compared with normal pancreas (n = 8). Results are expressed as fold over control. *, *P* < 0.05. C, immunohistochemistry of MK in selected patient tumors (5355, 5449, 5570, and 5691) vs. normal tissue. IgG control stainings revealed specificity of MK detection. D, Western blots showing steady-state MK expression in PDAC cell lines.
Gemcitabine-induced MK expression is dose dependent

Because all investigated cell lines are highly resistant to gemcitabine, we hypothesized that the MK overexpression may play a role in chemoresistance. To test this, we treated chemosensitive and chemoresistant lines with gemcitabine. Interestingly, we found that MK protein levels were increased in a dose-dependent manner in chemoresistant cells (Fig. 2A, top). In contrast, treatment of chemosensitive cells failed to increase MK protein levels (Fig. 2A, bottom). Moreover, MK protein levels were only barely detectable in control-treated as well as gemcitabine-treated chemosensitive cells. Because it is known that the MK signaling is post-translationally extinguished by proteasome-dependent degradation (22), we examined whether gemcitabine-induced MK upregulation occurs at the mRNA level. Indeed, we found that MK mRNA expression is dose-dependently induced by gemcitabine in chemoresistant PDAC cells (Fig. 2B, top). In contrast, MK mRNA levels in gemcitabine-treated chemosensitive cells remained either slightly decreased or unchanged (Fig. 2B, bottom). Moreover, we investigated whether other chemotherapeutics used in PDAC treatment may also increase MK expression. As expected, treatment of chemoresistant cells with 5-FU resulted in dose-dependent MK upregulation, whereas no induction was again observed in chemosensitive cells (Supplementary Fig. S1). Because MK is a secreted growth factor involved in paracrine/autocrine regulation of growth and differentiation, we investigated whether the MK secretion is increased in chemoresistant cells upon gemcitabine treatment. Indeed, we found robust and dose-dependently released MK protein levels in gemcitabine-treated cells (Fig. 2C, top), whereas no increased MK secretion was detectable in chemosensitive cells (Fig. 2C, bottom).

RNAi-mediated abrogation of MK induction by chemotherapy restores chemosensitivity

Because MK is induced at the mRNA and protein level in gemcitabine-treated chemoresistant cells, we hypothesized that MK mediates intrinsic chemoresistance. First, we examined whether RNAi-mediated MK downregulation can restore chemosensitivity in PDAC cells. Transient transfections of PANC-1 and PaCa 5061 with 2 different MK siRNAs decreased efficiently MK expression (Fig. 3A and B, right). We then analyzed the consequences of depleted MK during chemotherapy and carried out cell viability assays. Strikingly, we observed that MK depletion can effectively restore chemosensitivity in chemoresistant cells (Fig. 3A and B, left). Moreover, we carried out rescue experiments via exogenous treatment of MK-depleted PaCa 5061 and PANC-1 cells with rh-MK. Here, we observed substantially increased chemoresistance in rh-MK–treated MK-depleted cells, compared with untreated cells and chemoresistance reached almost similar levels as control-transfected cells (Supplementary Fig. S2). As we found the MK secretion to be induced by gemcitabine, we examined whether BxPC-3 and L3.6pl cells may acquire a chemoresistance phenotype through exogenous treatment of rh-MK. Serum-deprived cells were pretreated with rh-MK followed by treatment with increasing gemcitabine concentrations and then transferred to viability assays. Interestingly, pretreatment of L3.6pl cells with rh-MK resulted in substantially increased viability compared with untreated cells (Fig. 3C, left) whereas chemosensitivity of BxPC-3 cells was unaltered (Fig. 3C, right).

MK overexpression contributes to EMT in pancreatic cancer

To identify molecular alterations that mediate chemoresistance and to investigate whether the MK expression is involved in this mechanism, we obtained a chemoresistant subclone from chemosensitive L3.6pl cells, hereafter designated as L3.6pl-Res, through sequential treatment with increasing gemcitabine concentrations over time. In viability assays, L3.6pl-Res cells showed a substantial enhancement of chemoresistance toward gemcitabine. However, during the establishment of L3.6pl-Res cells, we observed morphologic changes toward spindle-shaped morphology and enhanced pseudopodia formation (Fig. 4A, right), whereas L3.6pl cells displayed a cobblestone epithelial morphology (Fig. 4A, left). As previously reported (23), the observed phenotypic changes suggested that L3.6pl-Res cells had undergone EMT over time. To further confirm these observations, we analyzed in more detail by Western blotting and real-time RT-PCR, whether the acquisition of gemcitabine resistance induced molecular changes consistent with EMT. As a hallmark of epithelial cells, we found elevated expression of the epithelial adhesion molecule E-cadherin in L3.6pl cells. In addition, β-catenin and γ-catenin protein levels were prominently expressed, suggesting that L3.6pl cells display an epithelial phenotype. In contrast, analysis of L3.6pl-Res cells revealed increased expression of mesenchymal markers vimentin, fibronectin, and α-SMA (Fig. 4B). Moreover, expression of the EMT-related transcription factors Snail and NF-kB was markedly increased. Because Notch-2 is upregulated in gemcitabine-resistant cells (15), we also analyzed its expression and found substantially increased Notch-2 protein levels in L3.6pl-Res cells, suggesting that its expression is not only important for gemcitabine-induced EMT but also for acquired chemoresistance in PDAC. Analysis of L3.6pl-Res cells for other Notch family members did not reveal any and found no increased expression except for Notch-2 (Supplementary Fig. S3). Interestingly, gemcitabine treatment of chemosensitive BxPC-3 cells failed to induce Notch-2 expression (Supplementary Fig. S4). We then investigated whether MK is reexpressed in L3.6pl-Res cells and found tremendous MK mRNA as well as protein levels compared with L3.6pl cells (Fig. 4B and C). To pinpoint whether these molecular changes were due to changes in gene expression, we quantified the expression of E-cadherin, vimentin, Notch-2, and MK by using real-time RT-PCR (Fig. 4C). E-cadherin was greatly decreased whereas vimentin, MK, and Notch-2 expression was highly increased in L3.6pl-Res cells. Interestingly, these results were independently confirmed by the expression data obtained for 3 chemoresistant PDAC cell lines (data not shown).

In proof-of-concept experiments, we depleted MK expression in L3.6pl-Res cells by using 2 different siRNAs and treated cells with increasing gemcitabine concentrations followed by viability assays. MK-depleted L3.6pl-Res cells showed...
substantially decreased chemoresistance and reached almost similar levels of chemosensitivity as L3.6pl cells whereas si-control transfections had no influence on chemoresistance (Fig. 4D). We also carried out rescue experiments and treated MK-depleted L3.6pl-Res cells with exogenous rh-MK. As expected, chemoresistance is substantially increased in

Figure 2. Dose-dependent MK expression in gemcitabine-treated resistant PDAC cells. A, chemoresistant and sensitive PDAC lines were treated with increasing gemcitabine concentrations (0.25–10 μmol/L) or left untreated (C). MK protein expression was assessed by using specific antibodies. B, real-time RT-PCR was conducted to analyze whether increased MK protein levels were linked to higher MK gene expression in chemoresistant and sensitive lines. Cells were treated with increasing gemcitabine concentrations or left untreated (C) followed by RNA extraction. (P < 0.05). C, MK secretion is dose-dependently increased in gemcitabine-treated chemoresistant cells verified by immunoblotting of conditioned cell culture media.
whether EMT was reversed to MET in MK and/or Notch-2

Consequently, we verified by real-time RT-PCR and Western blotting expression compared with si-control (Fig. 5A and B). Subsequently, we found elevated E-cadherin and dramatically decreased vimentin protein expression in siRNA-transfected L3.6pl-Res cells, which is consistent with the reversed transition from EMT to MET (Fig. 5A). To validate whether Notch downstream signaling is affected (Fig. 5A). It is well known that an increased migration/invasion potential is a hallmark of mesenchymal cells. Therefore, we carried out migration/invasion assay by using MK-depleted PaCa 5061, 5072, 5156, and L3.6pl-Res cells or rh-MK-treated MK-depleted cells, compared with untreated cells (Supplementary Fig. S2). Our results suggest that gemcitabine-induced EMT accompanied by acquired chemoresistance is feasible in a subset of PDAC cells and involves the reexpression of MK and increased Notch-2 expression.

**MK depletion induces reversal of EMT to MET in L3.6pl-Res cells**

Because several growth factors have been shown to trigger EMT, we hypothesized that MK may play a role in the acquisition of EMT in PDAC. To confirm this, we decreased MK expression in L3.6pl-Res cells with siRNA and investigated mRNA and protein expression with respect to EMT markers. It was previously shown that depletion of Notch-2 in EMT-acquired cells induced the reversal to MET (15). We, therefore, decreased Notch-2 expression in L3.6pl-Res cells by using siRNA. As shown in Fig. 5, the applied MK and Notch-2 siRNAs were highly effective which resulted in decreased protein expression compared with si-control (Fig. 5A and B). Subsequently, we verified by real-time RT-PCR and Western blotting whether EMT was reversed to MET in MK and/or Notch-2-depleted L3.6pl-Res cells. Indeed, E-cadherin mRNA expression was significantly increased in either MK or Notch-2 siRNA-transfected L3.6pl-Res cells compared with si-control (Fig. 5C). To further confirm that si-MK and/or si-Notch-2-transfected cells are able to induce MET, we analyzed vimentin expression and found significantly decreased mRNA levels (Fig. 5C). Along with the mRNA expression, we found elevated E-cadherin and dramatically decreased vimentin protein expression in siRNA-transfected L3.6pl-Res cells, which is consistent with the reversed transition from EMT to MET (Fig. 5A). To validate whether Notch downstream signaling is affected in Notch-2–depleted cells, we analyzed the protein expression of NF-κB/RelA. On the basis of previously published observations (24), we found substantially decreased NF-κB protein expression in si-Notch-2–transfected cells, suggesting that Notch downstream signaling is affected (Fig. 5A). Interestingly, we found decreased NF-κB protein expression in si-MK–transfected L3.6pl-Res cells, suggesting that MK cross-talks with Notch to positively regulate Notch downstream signaling (Fig. 5A). It is well known that an increased migration/invasion potential is a hallmark of mesenchymal cells.
si-control – transfected cells. As expected, we observed that MK-depleted chemoresistant PDAC cells migrated and invaded significantly less than control-transfected cells in vitro (Supplementary Fig. S5). MK mediates chemoresistance through binding and activation of Notch-2

Because MK and Notch-2 expression is dramatically increased in chemoresistant PDAC cells and both are linked to EMT, we hypothesized that MK may trigger Notch signaling and subsequently the activation of antiapoptotic pathways such as NF-κB signaling to promote chemoresistance in PDAC. We first analyzed a possible interaction between MK and the extracellular domain of Notch-2 by subcloning coding region for MK into pCS2 (pCS2-MK) and extracellular domain (residues 1–351) of Notch-2 (Notch-2-exD1–351) into p3xFLAG-CMV-14 resulting in expression of Flag-tagged Notch-2-exD1–351. The Flag-Notch-2-exD1–351 expression construct was transiently cotransfected along with pCS2-MK into PANC-1 cells, and protein complexes were precipitated with antibodies against MK followed by immunoblotting for detection of Flag-tagged Notch-2-exD1–351 and vice versa. Appropriate control transfections were carried out by cotransfections of pCS2-MK along with p3xFLAG-CMV-14. Strikingly, we found robust interaction of MK and Notch-2-exD1–351 in PANC-1 cells, whereas MK failed to interact with Flag alone (Fig. 6A, left). The same experiment was done vice versa and revealed positive interaction between MK and Notch-2-exD1–351 (Fig. 6A, right). To investigate whether the MK–Notch-2 interaction is functional with respect to Notch-2 activation which is followed by the γ-secretase–mediated cleavage of Notch-2ICD, we treated chemoresistant cells with rh-MK and analyzed cells for the presence of Notch-2ICD with specific antibodies. Treatment of chemoresistant cells with rh-MK resulted in an intensified cleavage of Notch-2ICD compared with untreated (control) cells, whereas full-length Notch-2

Figure 4. MK expression is linked to morphologic and molecular changes consistent with EMT. A, L3.6pl cells display cobblestone epithelial morphology (left), whereas L3.6pl-Res cells display spindle-shaped morphology with loss of polarity and enhanced pseudopodia formation (right; ×20). B, epithelial and mesenchymal markers in L3.6pl and L3.6pl-Res cells. Note reexpression of MK and increased Notch-2 expression in resistant cells. C, real-time RT-PCR was used to quantify gene expression of E-cadherin, vimentin, Notch-2, and MK in L3.6pl and L3.6pl-Res cells. **, P < 0.01; *, P < 0.05. D, L3.6pl-Res cells were transfected with 2 different MK siRNAs or si-control. Then, cells were treated with increasing gemcitabine concentrations for 24 hours, and cell viability was measured. Untransfected L3.6pl and L3.6pl-Res cells were also subjected to MTT assay. Left, RNAi-mediated MK depletion is linked to substantially increased chemosensitivity in L3.6pl-Res cells compared with si-control. Right, RNAi-mediated downregulation of MK protein levels.
protein was slightly decreased (Fig. 6B). We found that rh-MK–mediated cleavage of Notch-2ICD was dose independent and efficiently induced Notch-2 intracellular cleavage in the same manner. We then examined whether the rh-MK–mediated increase of Notch-2ICD could force up NF-kB protein expression which is known to be the central regulator of antiapoptotic pathways, and found elevated NF-kB protein levels in Notch-2–activated cells. To further validate MK-mediated Notch-2 cleavage and activation, we analyzed protein expression of the well-known Notch target in mammals, Hes-1, and found elevated protein levels in rh-MK–treated cells (Fig. 6B).

Because it was recently shown that elevated Notch-1 expression is linked to increased resistance against oxaliplatin in colorectal cancer (16), we analyzed whether RNAi-mediated Notch-2 depletion would affect chemoresistance in PDAC. We transiently transfected PaCa 5061 and PANC-1 cells with either si-control or si-Notch-2 and treated cells with gemcitabine (5 μmol/L) or left them untreated (Mock) followed by viability assays. The combination of si-Notch-2 and gemcitabine decreased cell viability of both resistant lines compared with si-control–transfected cells (Fig. 6C; P < 0.05).

Discussion

Despite improved diagnostic tools for PDAC, the often existing unspecific and indistinct symptoms delay diagnosis and render the majority of cases nonresectable (25). Also, completely resected patients develop recurrent disease and eventually require palliative treatment. The majority of patients receive little or no benefit from adjuvant therapies mainly because most of the cancer cells have been shown to be either intrinsically chemoresistant or become chemoresistant during therapy.

In this study, we addressed the evident need of identifying novel players and sought to further comprehend their molecular role for chemoresistance in PDAC. It was recently shown that MK secretion has cytoprotective functions in hepatocytes against cellular damage when exposed to heavy metals or injury in the heart (26, 27). The external stimuli and intracellular signaling that may promote MK expression are unknown. Several receptors have been proposed to turn activated upon MK binding in embryonic tissue as well as in cancer. MK was shown to bind low-density lipoprotein receptor–related protein, α5β1-integrin, and α6β1-integrin in embryonic neuronal tissue which mediated intracellular phosphorylation of Src family proteins (28, 29). For promotion of migration and invasion of human head and neck cancer cells, it was proposed that MK binds to tetraspanin, which then induces tyrosine phosphorylation of focal adhesion kinase followed by activation of the STAT1 pathway (30). In this study, we have found a robust interaction of MK and Notch-2 in vitro. The treatment

![Diagram](cancerres.aacrjournals.org)
of Notch-2–positive PDAC cells with soluble MK resulted in Notch-2 activation and was linked to upregulation of Notch downstream targets, Hes-1 and NF-κB/RelA, suggesting that the MK–Notch-2 interaction positively regulates Notch downstream signaling. Notch-2 and Notch-4 expression were recently described to be upregulated in chemoresistant PDAC which is linked to acquired EMT (15). Our data confirmed this in the case of Notch-2 but not for Notch-4. Although all Notch receptors are more or less expressed in the investigated cells, we observed a specific Notch-2 upregulation suggesting a *sine qua non* in triggering and maintaining the EMT phenotype which is accompanied by increased chemoresistance. In proof-of-concept studies, we found substantially increased chemosensitivity in Notch-2–depleted cells, suggesting that Notch signaling not only contributed to proliferation and differentiation but also to the regulation of cell death through cross-talking with NF-κB signaling. Our observations are, therefore, in agreement with previous studies showing Notch signaling to positively influence NF-κB signaling through the transcriptional regulation of positive regulators of the pathway in T-cell leukemia (31). In this context, it was shown that NF-κB is not only frequently constitutive active in PDAC but also represses E-cadherin expression in mammary epithelial cells and, thus, supporting EMT (32, 33).

According to recent publications, the natural Notch ligand Jagged-1 is upregulated in chemoresistant PDAC (15) and chemoresistant colorectal cancer (16). Obviously, downregulation of Jagged-1 expression in colorectal cancer revealed no functional relevance for chemoresistance (16). Interestingly, we also observed that Jagged-1 depletion in PDAC cells had no impact on chemoresistance (data not shown) suggesting that binding or recruitment of other molecules are of pivotal importance for mediating acquired chemoresistance in PDAC.

It is well known that the phenotype of chemoresistant- and EMT-acquired PDAC cells is associated with changes in their morphology. Through serial gemcitabine exposure of L3.6pl cells, we also observed morphologic changes that were associated with decreased expression of epithelial markers and an increase of mesenchymal markers suggesting that L3.6pl cells were capable of inducing an EMT phenotype over time. Moreover, we found reexpression of MK and increased Notch-2 expression in EMT-acquired L3.6pl-Res cells. Attempts to establish a drug-resistant cell clone from BxPC-3 cells were unsuccessful. This phenomenon was also
mirrored by the observation that exogenous treatment of L3.6pl cells with rh-MK resulted in substantially increased chemoresistance whereas chemosensitivity of BxPC-3 was unaltered. Therefore, we hypothesized that cells lacking corresponding signaling cascades or a specific set of molecules are not capable of undergoing EMT accompanied by increased chemoresistance in PDAC. Interestingly, it was already shown that MK and Notch-2 are independently involved in EMT processes. During early organogenesis in mice, the MK expression was shown to be associated with epithelial–mesenchymal interactions. The authors observed that the MK gene was highly expressed in mesenchyme whereas the protein was distributed to the surface of epithelial cells which expressed nondetectable mRNA amounts during several stages of tooth development in mice suggesting that MK may act by a paracrine mechanism (34). Strikingly, it was shown that Notch-2 expression is also regulated by epithelial–mesenchymal interactions in the developing mouse tooth and is reexpressed in adults under pathologic conditions suggesting that Notch-2 is involved in cellular repair processes during vertebrate development (35).

Our data are also in full agreement with previous observations in which MK was identified as being overexpressed in chemoresistant gastric cancer cells (36). Factors that may potentiate transcription or reexpression of MK are still unknown. Further, one could speculate that increased MK secretion may also trigger intercellular chemoresistance by activating Notch signaling in surrounding neighboring cells in order to activate defense mechanisms against drug toxicity. It is probable that our results may also explain previous observations in which the treatment of chemosensitive cells with conditioned cell culture media from MK-overexpressed cells was linked to increased Akt phosphorylation and, thus, increased cell viability (37).

In conclusion, our study revealed the identification of a novel mediator of EMT and drug resistance in PDAC cells. To further corroborate our findings in vivo, knowledge transfer to mouse models is of pivotal importance. It should, further, be determined whether these findings are applicable to other cells capable of developing resistance toward other chemotherapeutics. Therefore, targeting MK or blocking/reversing EMT prior to or during chemotherapy may force chemoresistant cells to revert to sensitive cells and, thus, may eventually be of tremendous benefit for patients suffering from advanced chemoresistant cancers, as it is unfortunately still the reality for PDAC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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