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WNT5A-NFAT Signaling Mediates Resistance to Apoptosis in Pancreatic Cancer[1,2]

Heidi Griesmann*, Stefanie Ripka*, Moritz Pralle*, Volker Ellenrieder*, Sandra Baumgart*, Malte Buchholz*, Christian Pilarsky†, Daniela Aust‡, Thomas M. Gress* and Patrick Michl*

*Department of Gastroenterology and Endocrinology, University Hospital, Philipps-University, Marburg, Germany; †Department of Surgery, University Hospital "Carl Gustav Carus", Technical University, Dresden, Germany; ‡Department of Pathology, Technical University, Dresden, Germany

Abstract

INTRODUCTION: WNT5A belongs to the Wnt family of secreted signaling molecules. Using transcriptional profiling, we previously identified WNT5A as target of the antiapoptotic transcription factor CUX1 and demonstrated high expression levels in pancreatic cancer. However, the impact of WNT5A on drug resistance and the signaling pathways employed by WNT5A remain to be elucidated. *OBJECTIVES:* This project aims to decipher the impact of WNT5A on resistance to apoptosis and the signaling pathways employed by WNT5A in pancreatic cancer. *METHODS:* The impact of WNT5A and its downstream effectors on tumor growth and drug resistance was studied *in vitro* and in xenograft models *in vivo*. Tissue microarrays of pancreatic cancer specimens were employed for immunohistochemical studies. *RESULTS:* Knockdown of WNT5A results in a significant increase in drug-induced apoptosis. In contrast, overexpression of WNT5A or addition of recombinant WNT5A mediates resistance to apoptosis *in vitro*. In our attempt to identify downstream effectors of WNT5A, we identified the transcription factor nuclear factor of activated T cells c2 (NFATc2) as transcriptional target of WNT5A signaling. NFATc2 confers a strong antiapoptotic phenotype mediating at least in part the effects of WNT5A on drug resistance and tumor cell survival. *In vivo*, WNT5A expression leads to resistance to gemcitabine-induced apoptosis in a xenograft model, which is paralleled by upregulation of NFATc2. Both WNT5A and NFATc2 proteins are highly expressed in human pancreatic cancer tissues and their expression levels correlated significantly. *CONCLUSION:* We identified the WNT5A-NFATc2 axis as important mediator of drug resistance in pancreatic cancer.

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Abbreviations: NFAT, nuclear factor of activated T cells; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; MTA, multiple tissue array; siRNA, small interfering RNA; PARP, poly(ADP-ribose) polymerase

Address all correspondence to: Priv.-Doz. Dr Patrick Michl, Department of Gastroenterology, University of Marburg, Baldinger Strasse, D-35043 Marburg, Germany. E-mail: michlp@med.uni-marburg.de

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[2]This article refers to supplementary materials, which are designated by Figures W1 to W9 and are available online at www.neoplasia.com.

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Introduction

The Wnt family of proteins are secreted cysteine-rich glycoproteins comprising 19 highly conserved members [1] that have been shown to play a pivotal role in embryogenesis, development, and tissue homeostasis. Wnt proteins lack transmembrane domains, are posttranslationally modified by glycosylation [2], and are known to bind to the Frizzled family of transmembrane receptors [3]. Various *Wnt* genes are differentially expressed during embryogenesis and among various tissues, including stem cell compartments of several organs [4].

In addition to their crucial role in embryogenesis and development, several Wnt ligands have been linked to carcinogenesis and tumor progression: High levels of certain Wnt family members such as WNT1, WNT3A, and WNT7A are capable of transforming various cell types into tumor cells. Other Wnt ligands such as WNT4, WNT5A, and WNT6, however, fail to induce cellular transformation [5].

The role of WNT5A in tumorigenesis remains ambiguous. In hematopoietic cells as well as breast and renal cancers, WNT5A has been shown to inhibit tumor cell proliferation [6–8] and was associated with a good prognosis [9]. However, accumulating data in other tumor types indicate that increased WNT5A expression may actually promote cancer progression. For example, WNT5A has been demonstrated as potent enhancer of cell motility and invasiveness in melanomas [10] and is highly expressed in various cancers of the lung, stomach, and prostate [11–13].

The downstream signaling events induced by WNT5A also remain controversial and may be cell type–dependent [14]. Most Wnt members signal through Frizzled receptors and activate the *dishevelled* gene, resulting in the inhibition of glycogen synthase kinase-3 beta (GSK-3β) activity and the subsequent stabilization of its target β-catenin, which results in altered gene transcription. In contrast to this "canonical" pathway, other Wnt members may also signal through "noncanonical" pathways such as the Wnt-calcium pathway involving activation of phospholipase C, protein kinase C (PKC), and calmodulin-dependent protein kinase II (CaMKII) or the planar cell polarity pathway involving cytoskeletal reorganization and activation of Rac/Rho GTPases [14–17].

In many cell types, WNT5A has been described to signal through noncanonical Wnt signaling pathways, although it also has the potential to activate the canonical Wnt signaling pathway depending on the receptor context [14,16,17]. In addition to signaling through the Frizzled receptors, WNT5A may activate receptor tyrosine kinases such as Ror2 and Ryk, which are able to inhibit canonical Wnt signaling [18].

Interestingly, in several cell types such as osteoblasts, mammary epithelial cells, and endothelial cells, a link between WNT5A and the nuclear factor of activated T cells (NFAT) transcription factor family has been proposed [9,19,20]. The NFAT family comprises four members of calcium/calcineurin-regulated proteins particularly recognized for their central roles in gene regulation during T-lymphocyte activation [21]. However, a multitude of studies have demonstrated that NFAT proteins are also expressed in cells outside the immune system, and emerging evidence indicates a key role for two NFAT members, NFATc1 and NFATc2, during carcinogenesis by regulating crucial aspects of neoplastic transformation and tumor progression [21]. Both isoforms are frequently overexpressed in epithelial malignancies, which is associated with a highly malignant phenotype [22].

Recently, we identified WNT5A as an important downstream effector of the homeodomain transcription factor CUX1, also known as CUTL1 [16]. We had shown before that CUX1 mediates tumor invasion and tumor cell survival in solid tumors including pancreatic cancer [23,24]. On the basis of these data, it was the aim of this study to characterize the role of the CUX1 target WNT5A on tumor cell survival and drug resistance and to decipher the signaling events downstream WNT5A.

Materials and Methods

Material and Cell Lines

ImimPC1 cells were kindly provided by F. X. Real (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain) [25]. The PaTu-8988t cell line was received from the German Collection of Cell Lines (DSMZ, Braunschweig, Germany). Both cell lines were derived from human primary ductal adenocarcinomas (PaTu-8988t) or liver metastases of pancreatic ductal adenocarcinomas (ImimPC1) and carry activating K-Ras mutations. Stable PaTu-8988t–NFATc2 cells are a kind gift from V. Ellenrieder (University of Marburg). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Darmstadt, Germany) supplemented with 10% fetal calf serum (Gibco) and 250 μg/ml gentamicin (PAA, Cölbe, Germany). The amphotropic packaging cell line LinX was maintained in Dulbecco's modified Eagle's medium, 10% fetal calf serum, gentamicin, and 100 μg/ml hygromycin B (Roth, Karlsruhe, Germany). All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. Gemcitabine was obtained from Lilly (Bad Homburg, Germany) and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and rWnt5A from R&D Systems (Wiesbaden-Nordenstadt, Germany). Reagents were used at the following working concentrations: TRAIL, 15 ng/ml; gemcitabine, 80 μg/ml; rWNT5A, 500 ng/ml; doxycycline (Sigma-Aldrich, Taufkirchen bei München, Germany), 4 μg/ml; puromycin (Sigma), 1.25 μg/ml; G418 (PAA), 100 μg/ml.

Plasmids and Small Interfering RNA

The open reading frame (ORF) of hWNT5A was amplified by polymerase chain reaction (PCR) from the human cDNA clone MGC:71588 IMAGE:30346200 and cloned into the Tet-repressible expression vector pBig2r [26], which was a kind gift of J. R. Hall, using the *Kpn*I and *Spe*I restriction sites. The pBig2r-WNT5A and empty pBig2r vectors were transfected into PaTu-8988t cells. For generation of stable clones, cells were cultured in the presence of 400 μg/ml hygromycin B. Stable expression of WNT5A was confirmed after incubation with doxycycline (4 μg/ml for 24 hours) through immunoblot analysis. Stable ImimPC1 cells were generated using a retroviral system. For retroviral expression, WNT5A was amplified and cloned into pENTR vector using pENTR/D-TOPO Cloning Kit (Invitrogen, Darmdstadt, Germany) and recombined into a Gateway competent pQCXIP vector (kind gift from T. Stiewe, University of Marburg). The luciferase reporter constructs pNFAT and *cis*NFAT were kindly provided by V. Ellenrieder (University of Marburg). The pGL3-Enhancer as control was purchased from Promega (Mannheim, Germany). Small interfering RNA (siRNA) purchased from Ambion (Darmstadt, Germany) against WNT5A or NFATc2 was transfected at a final concentration of 10 nM using RNAiMAX (Invitrogen) according to the manufacturer's protocol. All results could be verified by two independent silencing sequences.

Retroviral Infection

To produce retroviruses, we transfected LinX packaging cells with 5 μg of retroviral vectors; 48 and 72 hours after transfection, the retrovirus-containing supernatant was harvested, filtered, and supplemented with 8 μg/ml polybrene (Sigma). Target cells were transduced by spin infection at 1500 rpm, 37°C for 1 hour and selected with puromycin and G418.

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