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Usp9x Promotes Survival in Human Pancreatic Cancer and Its Inhibition Suppresses Pancreatic Ductal Adenocarcinoma *In Vivo* Tumor Growth[1]

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Abstract

Usp9x has emerged as a potential therapeutic target in some hematologic malignancies and a broad range of solid tumors including brain, breast, and prostate. To examine Usp9x tumorigenicity and consequence of Usp9x inhibition in human pancreatic tumor models, we carried out gain- and loss-of-function studies using established human pancreatic tumor cell lines (PANC1 and MIAPACA2) and four spontaneously immortalized human pancreatic patient-derived tumor (PDX) cell lines. The effect of Usp9x activity inhibition by small molecule deubiquitinase inhibitor G9 was assessed in 2D and 3D culture, and its efficacy was tested in human tumor xenografts. Overexpression of Usp9x increased 3D growth and invasion in PANC1 cells and up-regulated the expression of known Usp9x substrates Mcl-1 and ITCH. Usp9x inhibition by shRNA-knockdown or by G9 treatment reduced 3D colony formation in PANC1 and PDX cell lines, induced rapid apoptosis in MIAPACA2 cells, and associated with reduced Mcl-1 and ITCH protein levels. Although G9 treatment reduced human MIAPACA2 tumor burden *in vivo*, in mouse pancreatic cancer cell lines established from constitutive (8041) and doxycycline-inducible (4668) KrasG12D/Tp53R172H mouse pancreatic tumors, Usp9x inhibition increased and sustained the 3D colony growth and showed no significant effect on tumor growth in 8041-xenografts. Thus, Usp9x inhibition may be therapeutically active in human PDAC, but this activity was not predicted from studies of genetically engineered mouse pancreatic tumor models.

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Introduction

Pancreatic cancer is comprised of both exocrine and endocrine tumors of the pancreas with more than 90% of the pancreatic tumors classified as pancreatic ductal adenocarcinoma (PDA). PDA is relatively rare (11th most common) with 45,000 new cases per year but with a very poor prognosis [1]. Poor outcomes can be associated with the asymptomatic nature of early-stage disease, typically late clinical diagnosis, and high metastatic activity of the primary tumor resulting in a 5-year survival rate of only 6% [2,3]. Even in cases where surgical intervention of localized disease is possible, 5-year survival (20%) is not markedly improved, highlighting the high propensity of PDA to seed micrometastatic disease and underscoring the need to fully interrogate initiating and promoting lesions or

Abbreviations: PDA, pancreatic ductal adenocarcinomas; PanIN, pancreatic epithelial lesions; GFR, growth factor reduced; DOX, doxycycline; 3D, three-dimensional; Usp9x-OV, Usp9x overexpression; DUB, deubiquitinase

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pathways that may provide new approaches in detection and therapy [4]. Toward that goal, animal models built on common genetic lesions detected in PDA patients have been informative and have led to the reassessment of the role of specific gene mutations, such as Kras, in tumorigenicity [5]. The Kras oncogene is frequently (90%) mutated in pancreatic cancer, with G12D being the most common mutation with capacity to constitutively activate Kras GTPase [6,7]. Kras mutations occur in precursor lesions called the pancreatic epithelial lesions (PanIN), indicating an early role in the progression toward pancreatic cancer [6,8]. *In vivo* mouse models have established the role of oncogenic Kras in the initiation of pancreatic cancer in mice [9,10], while recent reports outline the importance of mutated Kras in pancreatic cancer maintenance [11]. Using a mouse model which allows for inducible, pancreas-specific, and reversible expression of oncogenic KrasG12D, with or without one allele of the tumor suppressor p53, Collins et al. showed that KrasG12D drives pancreatic tumorigenesis and is required for tumor maintenance [11]. However, KrasG12D induction alone causes only limited onset of tumorigenesis, which may reflect clinical observations which estimate that a single point mutation can occur 10 to 15 years prior to establishment of invasive disease and metastatic lesions [12]. Thus, complementation of Kras tumorigenicity with additional PDA-associated mutations reduces the latency of tumor development and provides useful PDA mouse models of human disease [12]. However, these models do not allow an unbiased assessment of other genes and epigenetic changes that may play a role in the emergence of invasive PDA [12]. This deficiency was recently addressed using insertional gene disruption technology provided by the "Sleeping Beauty" transposon [13,14]. Using this transposon to interrogate gene disruption associated with shortened latency in a KrasG12D pancreatic tumor model, Perez-Mancera et al. described several cooperative genes that were previously described in PDA patients [13]. In addition, Usp9x, a DUB previously associated with tumor-permissive pathway control, was mapped as the most common insertionally disrupted gene in the KrasG12D background that cooperated in promoting KrasG12D tumorigenesis.

Usp9x has been described as a critical mediator of cell survival. Increased expression of Usp9x is associated with hematologic malignancies including follicular lymphoma, diffuse large B cell lymphoma, multiple myeloma [15], chronic myelogenous leukemia [16], as well as solid tumors such as brain tumors [17], esophageal squamous cell carcinomas [18], prostate [19] and breast cancers [15,20]. High expression levels of Usp9x associate with poor prognosis in multiple myeloma [15] and esophageal squamous cell carcinomas [18]. Some cancers, including primary breast cancer, demonstrate an association between Usp9x and Mcl-1, a prosurvival BCL2 family member that is essential for stem and progenitor cell survival and is known to confer chemo- and radioresistance in a variety of tumors including lymphoma, breast, renal, lung, bladder, and prostate cancer [18,21,22]. Inhibition of Usp9x has emerged as a therapeutic strategy in the treatment of hematologic malignancies, melanoma, and ERG-positive prostate tumors [15,19,23]. Usp9x inhibition is also shown to sensitize tumor cells to chemo- and radiotherapy by reducing Mcl-1 levels [21,22,24,25].

In the present study, we examined the role of Usp9x in pancreatic tumors. We established a 3D culture model of genetically engineered mouse tumor derived cell lines, established human pancreatic cancer cell lines, and patient-derived pancreatic cancer cell lines. Using these models, we assessed the *in vitro* pancreatic phenotype resulting from Usp9x overexpression as well as the consequence of short hairpin RNA (shRNA)–mediated Usp9x knockdown and small molecule–mediated inhibition on that phenotype. We performed parallel assessments in murine pancreatic tumor–derived cell lines established from mice with constitutive or doxycycline-inducible expression of KrasG12D and Tp53R172H. The results suggest that Usp9x serves as a tumor suppressor in genetically engineered mouse pancreatic tumors, as previously demonstrated. However, in established human pancreatic tumor cells, Usp9x supports tumor cell survival and the malignant phenotype, illustrating wide distinctions in function in murine tumor cell models and *bona fide* human pancreatic cancer while also highlighting the potential for Usp9x inhibitors to be used in the treatment of human PDAC.

Material and Methods

Reagents

All cell culture reagents and culture media were purchased from Invitrogen (Grand Island, NY). Growth factor reduced (GFR) Matrigel (# 354230) was purchased from BD Biosciences (Bedford, MA). Tet-free fetal bovine serum was from Clontech (Mountain View, CA). HA-UbVS was from Boston Biochem (Cambridge, MA). Doxycycline (DOX), Puromycin, MTT, and other laboratory reagents were from Sigma (St. Louis, MO). EOAI3402143 (or G9) was synthesized and purified by Evotec Ltd. (Abingdon OX14 4RZ, United Kingdom).

Cell Culture and Establishment of Stable shRNA KD and Usp9x Overexpressing Cell Lines

All the established cell lines were procured from ATCC more than a year ago, and early passage cells were frozen in 10% DMSO complete media in liquid nitrogen following standard protocols for future use. Cell lines were authenticated by morphology and growth characteristics and were tested for mycoplasma. These cells were grown under standard culture conditions of 5% CO_2 at 37°C following ATCC recommendations. Murine cell lines 8041, 4668, 65671, and 13342 were obtained from our collaborator Marina Pasca Di Magliano; have been well characterized for morphology, growth characteristics, and xenograft studies [26]; and were grown in 10% FBS-RPMI. The 4668 cell line was grown in the presence of 1 μg/ml of DOX unless DOX depletion was required to inhibit Kras expression/activity. To avoid induction of Kras from basal DOX levels in fetal bovine serum, DOX depletion experiments were carried out using tetracycline-free serum.

The human patient-derived cell lines UM-2, UM-6, UM-16, and UM-76 were established in the laboratory of Diane Simeone by xenotransplantation of the human pancreatic adenocarcinomas into mice. Briefly, after surgical removal, the tumor was digested using the Tissue Dissociation Kit (Miltenyi Biotec, #130-095-929) and were subjected to single cell isolation using Magnetic Cell Isolation following MACS technology (Miltenyi Biotec, Gladbach, Germany, # 130-090-753, #130-093-235, and #130-093-237) as instructed by the manufacturer. The cells were plated and subcloned in 10% RPMI medium. Cultures were purified of mouse stroma by labeling them with a biotin-labeled anti-H2Kd mouse antibody (Southern Biotech, Alabama, USA, #1911-08) and removing them using anti-biotin beads (Miltenyi Biotec Gladbach, Germany, #130-090-485) on a magnetic bead column (Miltenyi Biotec, Gladbach, Germany, #130-042-901).

The lentiviral expression system for shRNA against human Usp9x was kindly provided by Dr. Dzwokai Ma (University of California, Santa Barbara), and production of virus and infection were