Restricted Heterochromatin Formation Links NFATc2 Repressor Activity With Growth Promotion in Pancreatic Cancer

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BACKGROUND & AIMS: Transcriptional silencing of the $p15^{INK4b}$ tumor suppressor pathway overcomes cellular protection against unrestrained proliferation in cancer. Here we show a novel pathway involving the oncogenic transcription factor nuclear factor of activated T cells (NFAT) c2 targeting a $p15^{INK4b}$-mediated failsafe mechanism to promote pancreatic cancer tumor growth. **METHODS:** Immunohistochemistry, real-time polymerase chain reaction, immunoblotting, and immunofluorescence microscopy were used for expression studies. Cancer growth was assessed in vitro by [^{3}H]thymidine incorporation, colony formation assays, and in vivo using xenograft tumor models. Protein-protein interactions, promoter regulation, and local histone modifications were analyzed by immunoprecipitation, DNA pull-down, reporter, and chromatin immunoprecipitation assays. **RESULTS:** Our study uncovered induction of NFATc2 in late-stage pancreatic intraepithelial neoplasia lesions with increased expression in tumor cell nuclei of advanced cancers. In the nucleus, NFATc2 targets the $p15^{INK4b}$ promoter for inducible heterochromatin formation and silencing. NFATc2 binding to its cognate promoter site induces stepwise recruitment of the histone methyltransferase Suv39H1, causes local H3K9 trimethylation, and allows docking of heterochromatin protein HP1γ to the repressor complex. Conversely, inactivation of NFATc2 disrupts this repressor complex assembly and local heterochromatin formation, resulting in restoration of $p15^{INK4b}$ expression and inhibition of pancreatic cancer growth in vitro and in vivo. **CONCLUSIONS: Here we describe a novel mechanism for NFATc2-mediated gene regulation and identify a functional link among its repressor activity, the silencing of the suppressor pathway $p15^{INK4b}$, and its pancreatic cancer growth regulatory functions. Thus, we provide evidence that inactivation of oncogenic NFATc2 might be an attractive strategy in treatment of pancreatic cancer.**

Keywords: Pancreatic Cancer; NFAT; $p15^{INK4b}$; Gene Silencing; Heterochromatin.

BASIC AND TRANSLATIONAL PANCREAS

Pancreatic ductal adenocarcinoma is among the most devastating human malignancies.[1] It is commonly diagnosed at advanced, already metastatic, and hence incurable stages. The poor prognosis is largely attributable to its aggressive biology and the propensity of tumor cells to rapidly grow and disseminate into distant organs. The current model of pancreatic carcinogenesis suggests that invasive ductal adenocarcinomas arise from histologically well-defined noninvasive lesions, called pancreatic intraepithelial neoplasias (PanINs), through a series of genetic, epigenetic, and signaling events that eventually overcome cell autonomous tumor suppressor mechanisms to promote carcinogenesis.[2,3]

One of the most important and potent antitumor mechanisms in mammalian cells is mediated by the INK4/ARF family of $p15^{INK4b}$, $p16^{INK4a}$, and $p19^{ARF}$ (mouse homologue to human $p14^{ARF}$) tumor suppressor genes.[4] Whereas $p19^{ARF}$ primarily works through inhibition of MDM2-mediated p53 destabilization, $p15^{INK4b}$ and $p16^{INK4a}$ proteins guard against unrestrained proliferation through inhibition of cyclin-dependent kinases 4 and 6, thus maintaining retinoblastoma family proteins in a hypophosphorylated, growth suppressive state.[5] The induction of INK4 proteins therefore allows normal cells to rapidly respond to cellular stress signals, DNA damage, and oncogenic signaling activation with induction of a G_1 growth arrest.[4] Depending on the mode, magnitude, and duration of a cell signal, $p15^{INK4b}$ and $p16^{INK4a}$ expression can cause a transient G_1 cell cycle arrest or a permanent state of senescence. However, during development and progression of pancreatic cancer, INK4 proteins are frequently silenced by genetic or epigenetic events.[6] Clearly, genetic deletion of $p16^{INK4a}$ or its silencing by CpG island hypermethylation are early events in pancreatic carcinogenesis already found with high frequency at stages of premalignant PanIN lesions.[6,7] In contrast, the $p15^{INK4b}$ tumor suppressor gene is rarely depleted by genetic inactivation or hypermethylation of its gene locus.[8] Rather,

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; CsA, cyclosporin A; H3K9-me, histone 3 lysine 9 trimethylation; HP, heterochromatin protein; NFAT, nuclear factor of activated T cells; PanIN, pancreatic intraepithelial neoplasia; PCR, polymerase chain reaction; siRNA, small interfering RNA.

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p15^{INK4b} expression and function are tightly controlled by mitogenic and antiproliferative stimuli; hence, silencing of its expression during cancer progression might originate from signaling-induced promoter repression.

Here we report a novel mechanism regulating the silencing of p15^{INK4b} in pancreatic cancer cells involving the oncogenic transcription factor nuclear factor of activated T cells (NFATc2). We show ectopic expression of this calcium/calcineurin responsive transcription factor in early precursor lesions and with increased frequency and intensity in tumor cells of advanced stages that is accompanied by silencing of the p15^{INK4b} tumor suppressor pathway.[9] On activation, nuclear NFATc2 targets the histone methyltransferase Suv39H1 to the p15^{INK4b} promoter for local histone H3K9 trimethylation. This creates a platform for heterochromatin protein (HP) 1γ binding and leads to promoter-restricted transition of accessible (open) euchromatin to compacted (closed) and transcriptionally silenced heterochromatin. Conversely, inactivation of NFATc2 prevents p15^{INK4b} promoter silencing and instead restores expression of this master tumor suppressor in cancer cells in vitro and in vivo. Together, this report describes a novel mechanism of NFATc2-mediated gene regulation and identifies a functional link between its previously unappreciated transcriptional repressor activity and silencing of the p15^{INK4b} tumor suppressor pathway in pancreatic cancer. In addition, we provide evidence that inactivation of NFATc2 might be an attractive strategy to reactivate antitumor defense mechanisms in pancreatic cancer.

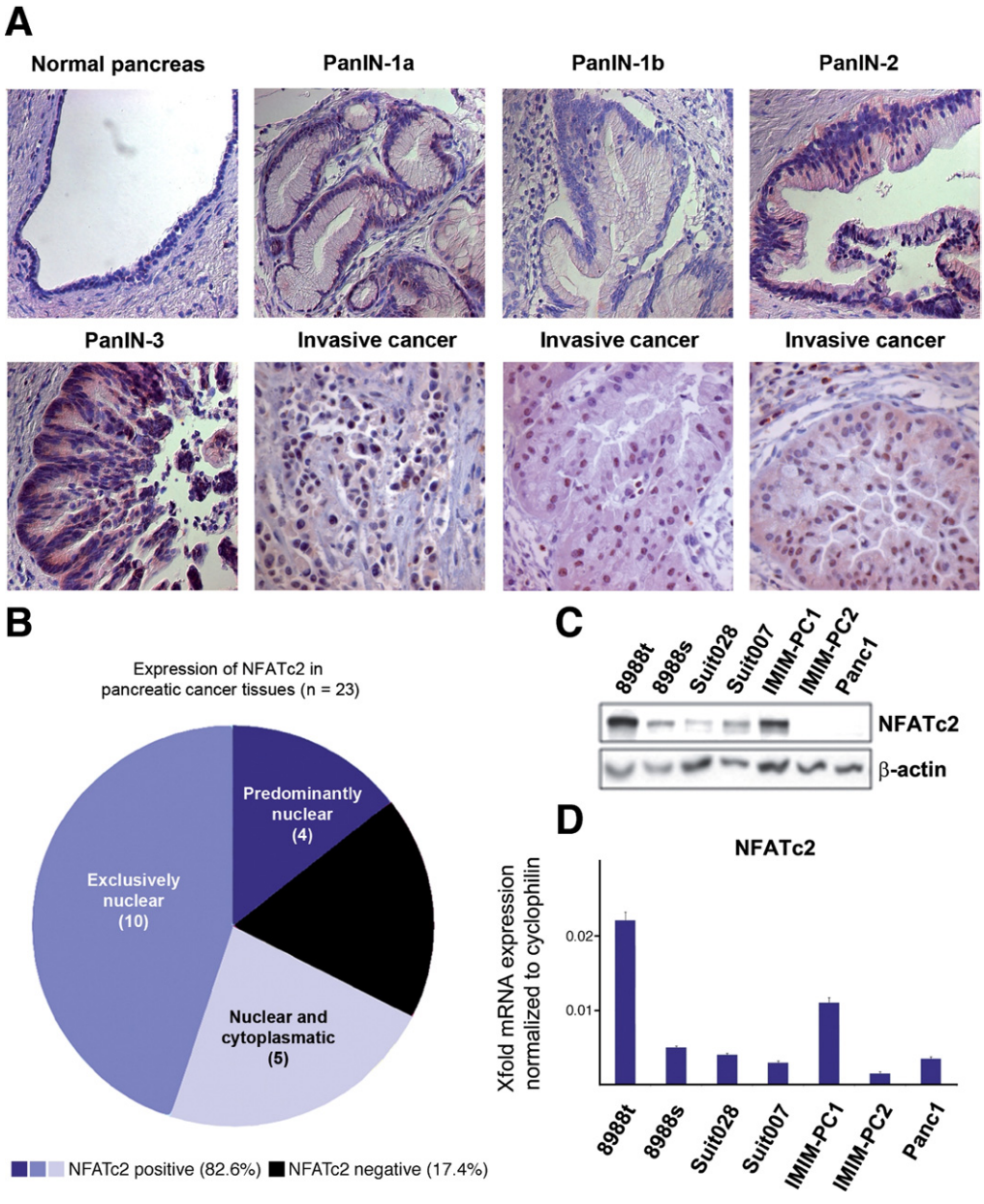

Figure 1. NFATc2 is widely expressed in human pancreatic cancer. (*A*) Immunohistochemical stainings of NFATc2 in sections of human pancreatic tissues to show that overexpression of NFATc2 is induced in PanIN-2 lesions and is highest in invasively growing cancers. (*B*) Classification of pancreatic cancers according to NFATc2 expression and localization. (*C*) Western blot analysis illustrates NFATc2 expression levels in different epithelial pancreatic cancer cell lines. (*D*) Quantitative real-time PCR to analyze the NFATc2 messenger RNA expression levels in different pancreatic cancer cells. The human cyclophilin gene was used as housekeeping gene control. Highest expression levels of NFATc2 were detected in PaTu8988t and IMIM-PC1 cells.