Primers on Molecular Pathways –
The NFAT Transcription Pathway in Pancreatic Cancer

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Abstract

The calcineurin-responsive nuclear factor of activated T cells (NFAT) family of transcription factors was originally identified as a group of inducible nuclear proteins, which regulate transcription during T lymphocyte activation. However, following their initial discovery, a multitude of studies quickly established that NFAT proteins are also expressed in cells outside the immune system, where they participate in the regulation of the expression of genes influencing cell growth and differentiation. Ectopic activation of individual NFAT members is now recognized as an important aspect for oncogenic transformation in several human malignancies, most notably in pancreatic cancer. Sustained activation of the Ca\textsuperscript{2+}/calcineurin/NFAT signaling pathway has emerged as a powerful regulatory principle governing pancreatic cancer cell growth. Activated NFAT proteins form complexes with key oncogenic proteins to regulate the transcription of master cell cycle regulators and proteins with functions in cell survival, migration and angiogenesis. This review pays particular attention to recent advances in our understanding of how the NFAT transcription pathway controls gene expression during development and progression of pancreatic cancer.

Introduction

The NFAT family, first described as a regulator of T cell activation and differentiation, is composed of four calcium-responsive isoforms named NFATc1 (NFAT2/NFATc), NFATc2 (NFAT1/NFATp), NFATc3 (NFAT4/NFATx), and NFATc4 (NFAT3) \cite{1}. Since their discovery more than two decades ago, it has become increasingly clear that NFAT transcription factors are operative not only in T cells but control critical processes in many vertebrate developmental systems \cite{2–5}. Emerging evidence also suggests a complex and predominant role of two isoforms, NFATc1 and NFATc2 in carcinogenesis, in which they regulate key aspects of neoplastic transformation and cancer progression \cite{6, 7}. Both isoforms are frequently overexpressed and active in gastrointestinal malignancies including pancreatic cancer, and are associated with a highly malignant and aggressive phenotype \cite{8, 9}. In the last few years, substantial progress has been achieved in...
our understanding of how NFAT factors are regulated in cancer cells and how their transcriptional activities contribute to pancreatic carcinogenesis.

**NFAT – Structural Features**

The predicted primary structure of NFAT is shown schematically in figure 1a. Two major regions of sequence homology, comprising the Rel-like DNA-binding domain (DBD) and the NFAT homology region (NHR), are represented in both isoforms. The DNA-binding domain, which is located between amino acid residues \( \approx 400 \) and \( \approx 700 \), is highly conserved within the NFAT family and shows moderate sequence similarity to the DNA-binding domains of Rel family proteins. The \( \approx 300 \) amino acids spanning NFAT homology region (NHR) is encoded in a single exon in all NFAT proteins and is located N-terminal to the DNA-binding domain [10]. The NHR is heavily phosphorylated in resting cells, with the phosphorylated serine residues distributed among conserved serine-rich sequence motifs termed SRR1–3 and SPI–3 regions [11]. The NHR domain also contains a critical nuclear localization sequence (NLS), a cofactor binding transactivation domain (TAD) and two calcineurin docking regions.

**Fig. 1.** a The NFAT structure. The domains depicted are retained in NFATc1 and NFATc2 isoforms. The N-terminal homology region (NHR) comprises the transactivation domain (TAD), the calcineurin docking sites (a, b), the nuclear localization sequence (NLS), the serine-rich regions SRR1–3 and the serine/proline-rich motifs SP1–3. Blue circles indicate phosphate groups in the SRR and SP domains that are removed by calcineurin. The red circle in the SRR-2 region indicates a calcineurin independent phosphorylation region. RHR = Rel-like homology region. b The NFAT regulatory loop. A rise in intracellular Ca\(^{2+}\) levels leads to stepwise activation of the phosphatase calcineurin, which dephosphorylates multiple phosphoserines on NFAT proteins, leading to the rapid translocation from the cytoplasm into the nucleus. NFAT proteins bind DNA target sequences in cooperation with partnering transcription factors (TF). Nuclear export is induced by rephosphorylation of the N-terminus of NFAT proteins by specific kinases such as GSK3β, CK1 and DYRK1. c Signal integration by NFAT and partner transcription factors. Activated NFAT factors rapidly bind to and induce transcription from a consensus (GGAAA–) promoter sequence localized within the serum responsive element of the c-Myc promoter. NFAT binding to this promoter region enhances the net histone acetylase activity associated with the c-Myc promoter and subsequently allows recruitment of a second Ras-MAPK signaling regulated transcription factor (ELK-1) to induce c-Myc expression.
NFAT – The Regulatory Circuit

In resting cancer cells, NFAT factors are located in the cytoplasm and in a highly phosphorylated, inactive state (fig. 1b). Ligand binding of many receptors results in the activation of phospholipase C (PLC), release of IP₃, and a transient release of Ca²⁺ from intracellular stores through IP₃ receptors. This initial release of Ca²⁺ is not sufficient to activate NFAT target genes in a number of cell types. Rather, an influx of Ca²⁺ through specialized Ca²⁺ release activated channels (termed CRAC) is required. CRAC provide the persistent Ca²⁺ signal that is necessary for sufficient calcineurin activation and the subsequent dephosphorylation of cytoplasmic NFAT proteins. Four cellular inhibitors of calcineurin complexes have been identified, all of which are able to block nuclear translocation of NFAT proteins: the calcineurin B homolog, CHP [12], the calcineurin activator CABIN [13, 14], the AKAP79 scaffold protein [15], and, finally, members of the DSCR/MCIP/RCAN family of endogenous calcineurin inhibitors [7]. In case of sustained Ca²⁺ increase and calcineurin activation, the phosphatase targets and dephosphorylates moderately conserved serine rich motifs at the N-terminal homology region (NHR) of NFAT to unmask its nuclear localization signals [11]. Subsequently, NFAT proteins shuttle into the nucleus where they accumulate on target promoter sites [16, 17]. Efficient dephosphorylation requires a successful docking interaction between NFAT and calcineurin [18–20]. The major docking site for calcineurin is located at the N-terminus of the NFAT regulatory domain with the consensus sequence PxIxIT. Current studies suggest that NFAT nuclear localization is accompanied by a relocalization of calcineurin from the cytoplasm into the nucleus. In fact, we found a nuclear co-localization of NFAT and calcineurin in tumor cells from early pancreatic cancer stages, and with a higher frequency and intensity in advanced stages of the disease [8].

NFAT – Transcriptional Mechanisms

Upon nuclear translocation, NFAT factors recognize the GGAAA consensus sequence within target promoter elements and bind DNA either as homodimers, heterodimers or through interaction with other transcription factors [9, 10]. In fact, NFAT proteins frequently cooperate with other transcription factors to elicit high-affinity binding on common target promoters. The best-documented example is the unrelated transcription factor activator protein-1 (AP-1; comprised of Fos-Jun complexes), which forms a quaternary complex with NFAT factors and DNA. AP-1 is the bona fide transcriptional partner for NFATs during T cell activation, but might also be involved in NFAT-regulated gene expression in carcinogenesis [4, 7]. In addition to AP-1, NFAT proteins cooperate with numerous other signaling regulated transcription factors with implications in cell activation, differentiation and adaptation such as GATA proteins, FoxP3 and members of the MEF family. Depending on the partners and cofactors they bind, NFAT factors can either enhance local chromatin acetylation and induce target promoter activation, or interact with histone deacetylases to silence target genes [21, 22]. In pancreatic cancer cells, NFATc1 and NFATc2 promote cell cycle progression and growth through complex formation with the ets-like factor Elk-1. Sequential chromatin immunoprecipitation analysis revealed that NFAT binding to the serum responsive element stimulates the histone acetyltransferase (HAT) p300 to create a hyperacetylated chromatin state at the proximal c-Myc promoter (fig. 1c). This event is required for the consecutive inducible recruitment of Elk-1 and the resulting induction of c-Myc expression following stimulation of the Ca²⁺/calcineurin pathway [22]. Disruption of this transcription complex prevents both c-Myc promoter transactivation and pancreatic cancer growth stimulation in vitro and in vivo. Thus, NFAT partnering proteins help control the specificity of NFAT promoter binding and the resulting mode of action.

Recently, additional transcription factors have been identified that form stable nuclear complexes with NFATc2 in pancreatic cancer cells. Among these factors is the signal transducer and activator of transcription-3 (Stat3) protein, a latent signal-induced transcription factor that has been implicated in numerous biological processes, including cell transformation and tumorigenesis [23, 24]. Like NFAT proteins, Stat3 is also regulated primarily at the level of its subcellular localization. In normal resting cells, Stat3 resides in a non-phosphorylated version in the cytoplasm. However, following cytokine and growth factor stimulation, Stat3 proteins are inducibly phosphorylated on a critical regulatory tyrosine residue (Y-705) that promotes their homodimerization and subsequent translocation into the nucleus where they control gene transcription.Clipstone’s group [25] has recently shown that Stat3 is activated in pancreatic cancer cells through an NFAT dependent autocrine factor. Moreover, genetic depletion of Stat3 attenuates the transformation capacity of NFATc1, suggesting a cooperative function of both factors in cancer. In line, we have most recently identified Stat3...
as one of a few inflammatory transcription factors that form nuclear complexes with NFATc2. The Stat3-NFAT interaction is tightly regulated on the level of phosphorylation and appears to be of outmost importance for NFATc1-induced cancer progression [Ellenrieder, unpublished results]. In addition to Stat3, NFAT recruits other signaling regulated transcription factors (e.g. Smad3, NF-kB, c-Myc and C/EBP) to integrate pathway specific signals to Ca\textsuperscript{2+}/calcineurin regulated transcription. Thus, structural and biochemical data indicate that NFAT transcription complexes function as signal integrators and detectors. One signal must be Ca\textsuperscript{2+}/calcineurin [26], while the second can be developmental or oncogenic such as the Ras-MAP kinase pathway [27]. Recent evidence also indicates that certain NFAT complexes might include transcriptional repressor proteins and members of the HDAC family of histone deacetylases that cooperate with the Ca\textsuperscript{2+}/calcineurin pathway to mediate gene silencing in non-transformed cells and in cancer as well. In osteoblasts, for instance, NFATc1 acts as a transcriptional co-repressor that sustains HDAC3 binding on the proximal region of the osteocalcin promoter in order to silence its activity and transcription [21]. Another good example is the NFATc1/C isoform that following sumoylation translocates to promyelocytic leukemia nuclear (PML) bodies, where it interacts with histone deacetylases to induce transcriptionally inactive chromatin at the interleukin-2 promoter [28]. This recent finding by the Serfling’s laboratory demonstrated for the first time that the modification by SUMO (small ubiquitin-like modifier) can convert NFATc1/C from an activator to a site-specific transcriptional repressor. Similar to sumoylation and phosphorylation, additional posttranslational modifications such as ADP-ribosylation can determine the mode and magnitude of NFAT transcription. In an elegant approach Olabisi et al. [29] have shown that poly-ADP-ribose polymerase PARP-1 binds to NFAT factors and induces ADP-ribosylation to increase its affinity for DNA binding. Thus, NFAT target gene selection and regulation is defined by the nuclear composition and selection of nuclear partner proteins and the presence and activity of NFAT modifying enzymes in the nucleus at a given time.

**NFAT – Termination of Transcription and Nuclear Export**

When Ca\textsuperscript{2+} entry is prevented or calcineurin activity is inhibited, nuclear import of the NFATc family is opposed by a highly efficient nuclear export mechanism (t\textsubscript{1/2} ≈ 15 min) that requires the sequential actions of a nuclear priming kinase, such as dual-specificity tyrosine kinase 1a (Dyrk 1a) [30, 31]. Notably, Dyrk1a phosphorylates nuclear NFAT, priming it for phosphorylation by GSK3\textbeta and subsequent nuclear export. Consequently, NFAT proteins accumulate in the cytosol. Both genetic and biochemical experiments indicate that in addition to Dyrk1a the protein kinase A (PKA) can serve as a priming kinase [30, 32]. It is unclear whether all NFAT proteins are phosphorylated by identical kinases and whether phosphorylation by GSK3\textbeta always results in a negative regulation. It also appears possible that the functional consequence of NFAT phosphorylation depends on the cell type and the nuclear context at a given time. For instance, we and others have most recently identified a novel and unexpected function of GSK3\textbeta in the regulation of NFATc2 in cancer cells. These data implicate a role for GSK3\textbeta mediated phosphorylation of the serine rich SP2 domain in protein stabilization and life-time regulation of NFATc2. GSK3\textbeta targets nuclear NFATc2 and protects the transcription factor from rapid ubiquitinylation and proteasomal degradation in cancer. It appears possible that additional modification will then contribute to successful NFAT export to the cytosol. Once in the cytosol, NFATc retention is controlled through maintenance kinases phosphorylating the unstructured N-terminus. These include c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK) and casein kinases [33, 34]. The fact that the N-termini are unstructured indicates that many kinases can probably phosphorylate them in vitro. To date, there is no genetic evidence that these kinases limit NFAT signaling, and, indeed, activation of MAPKs facilitates NFAT signaling rather than opposing it, as would be expected if they had a role in keeping NFATc in the cytoplasm.

**NFAT – Oncogenic Functions in Pancreatic Cancer**

One of the first studies implicating NFAT factors in cell proliferation was performed in fibroblasts, in which constitutively active NFATc1 induced cell transformation and colony formation [35]. Similarly, proliferation and anchorage-independent growth of pancreatic tumor cells is dependent on calcineurin activity and nuclear translocation of NFATc1, as previously shown by us [8]. This is consistent with the high levels of nuclear NFATc1 in pancreatic cancer cells and in particular in those cells with accelerated growth. Like NFATc1, the NFATc2 isoform is
also frequently overexpressed in solid tumors, including pancreatic adenocarcinoma [22], and in correlation with increased cell proliferation and invasion. Noteworthy, NFATc1 and NFATc2 might have redundant but also opposing functions in tumorigenesis. Tumor suppressor activities have recently been demonstrated for NFATc2 in fibroblasts, whereas NFATc1 was described in the same study as a strong oncogene able to transform cells [36]. In this recent study by Robbs et al. [36], constitutively active NFATc2 induced cell cycle arrest and inhibited H-Ras-induced transformation, whereas introduction of constitutively active NFATc1 showed opposite effects and caused increased proliferation and promotion of cell transformation. These observations underscore the idea that NFATc1 and NFATc2 partially share target specificity but also regulate a nonoverlapping subset of transcriptional targets, and therefore have nonredundant functions during cancer development and progression. However, whether or not NFAT proteins exert redundant or opposite effects in cancer cells might also depend on the origin of the cancer and the nuclear context given in a cell. Accordingly, we and others have reported strong growth promoting functions for both NFAT proteins in pancreatic, colon and breast cancer [6, 8, 22] and in particular in those with a highly invasive and metastatic phenotype. Upon stimulation, nuclear NFAT factors bind to and induce transcription from key cell cycle regulators such as CDK4, CDK6 and cyclin D resulting in accelerated cell cycle transition [35]. In addition, NFAT factors enhance the transcription of the proto-oncogene c-Myc and this event is a critical step in TGFβ-induced growth stimulation in pancreatic cancer [Ellenrieder, unpubl. results]. Mechanistically, TGFβ activates NFAT proteins to displace Smad3 repressor complexes from the TGFβ inhibitory element (TIE element) of the proximal c-Myc promoter and induce transcription of the oncogenic transcription factor [8]. Upon induction, c-Myc feeds back on NFAT to form complexes to induce growth promoting transcriptional programmes in cancer. Another hallmark of tumorigenesis is the ability of cancer cells to evade cell death. Programmed cell death by apoptosis occurs in virtually all cell types and is precisely regulated at cellular and molecular levels. NFAT proteins have shown to exert anti-apoptotic properties in numerous cancer types [7]. Overexpression of the NFATc1 or NFATc2 in adipocytes or lymphocytes protected cells from undergoing apoptosis in response to growth factor withdrawal [35] or following antigen stimulation, respectively. In pancreatic cancer cells, genetic depletion of either factor results in increased sensitivity to gemcitabine-mediated cell death. Together, these recent studies indicate that in addition to growth promotion, NFAT proteins contribute to tumor development by sustaining cell survival.

Besides growth stimulation and survival, NFAT proteins play key roles in migration and invasion of breast and pancreatic cancer cells [6]. The mechanisms by which NFAT factors function as pro-invasion transcription factors also lies within the transcriptional programme of genes that are induced in tumor cells. For instance, NFAT proteins induce the transcription of cyclo-oxygenase 2 (COX2) and of the genes encoding autotaxin, exonucleotide pyrophosphatase and phosphodiesterase 2 (ENPP2). Autotaxin is a secreted protein that converts lysophosphatidylcholine into lysophosphatidic acid (LPA), a potent mitogenic and motogenic factor for cancer cells [7].

COX2 catalyses the synthesis of prostaglandins such as prostaglandin E2 (PGE2) in tumor cells and in endothelial cells as well, leading to increased tumor cell migration and endothelial cell proliferation [37]. Moreover, knockdown of COX2 or treatment with COX inhibitors such as nonsteroidal anti-inflammatory drugs, rendered pancreatic cancer cells less invasive both in vitro and in vivo [37]. Thus, COX2 has emerged as a key enzyme in tumor cell migration and the metastatic dissemination of most human tumors, including pancreatic cancer cell infiltration to neighboring organ structures. A key step in pancreatic cancer cell migration and invasion is the induction and activation of matrix metalloproteinases such as MMP-2 [38]. In a genetic mouse model using highly metastatic osteosarcoma cells, a role of NFAT proteins in MMP-2 induction has been demonstrated [39]. Increased levels of MMP-2 expression and activity have also been reported in highly invasive and migrating pancreatic cancer cells and in particular in those with increased activation of TGFβ signaling [38]. Since NFATc1 and NFATc2 themselves are TGFβ-inducible transcription factors in pancreatic cancer cells, it will be interesting to elucidate their role in TGFβ-mediated gene expression during cancer progression. In pancreatic cancer, TGFβ has a dual function, working as a growth inhibitor at early tumor stages and a strong promoter of growth and migration in advanced stages of the disease. TGFβ then stimulates the expression of key cell cycle regulators (e.g. c-Myc and cyclin D) and promotes the induction of an epithelial-to-mesenchymal transdifferentiation, a hallmark of tumor cell migration and invasion [40]. Whether NFAT proteins contribute to TGFβ-driven carcinogenesis and whether they work independent or in concert with the Smad signaling molecules to mediate transcription in response to TGFβ is currently under investigation.
Conclusion

Taken together, growing evidence from biochemical, genetic and functional approaches suggest a multifunctional and powerful role for NFAT isoforms in pancreatic carcinogenesis. Based on recent key findings, NFATc1 and NFATc2 proteins are now being recognized as central regulators of gene transcription during tumor cell growth, survival and metastasis, therefore, making their pharmacological targeting an interesting goal in medical oncology.

However, the currently available pharmacological antagonists of calcineurin-NFAT signaling, such as FK506 and cyclosporin A (CsA) are not suitable for targeting the oncogenic function of NFAT. These potent inhibitors of NFAT dephosphorylation and nuclear accumulation, do not discriminate between NFAT and other downstream transcriptional components nor operate specifically in tumor cells. In fact, CsA and FK506 exert significant impact on the local and systemic immune response and in addition, induce severe toxic side effects such as neurotoxicity, nephrotoxicity and the development of high blood pressure.

Therefore, new treatment strategies that specifically switch off NFAT signaling in cancer cells without affecting the immune response in the tumor environment, are urgently needed.

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