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The Calcineurin-NFAT Pathway and Regulation of Pancreatic Function

Gurzegorz T. Gurda[1], Tegan St John[1], and John A. Williams[2]

[1]Department of Pathology, Gundersen Health System, La Crosse, WI and [2]Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI

e-mail: gtgurda@gundersenhealth.org

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1. General overview of the Calcineurin – NFAT pathway

Calcineurin (Cn), also known as protein phosphatase 2B, is a unique calcium/calmodulin activated serine/threonine phosphatase that serves as a calcium effector, usually requiring prolonged Ca^{2+} elevation, that alters both gene expression and cellular effectors (28). It was discovered in brain where it activates other phosphatases and neural proteins. Outside the brain, much of the gene regulation is mediated by NFAT (nuclear factor of activated T-cells). Other calcineurin substrates or binding partners include: the K^+ channel TRESK, KSR2 (kinase suppressor of Ras 2), AKAP79 (A-kinase anchoring protein 79), the transcription factors MEF2A and Crz1 and the binding protein Rcan1 (regulator of calcineurin 1). Calcineurin is specifically inhibited by immunosuppressant drugs such as cyclosporine A (CsA) and FK506 when bound to their intracellular receptors cyclophilin A and FK506-binding protein of 12 kDa (FKBP12), respectively. There are also several endogenous calcineurin binding proteins that inhibit its action, including Calsarcin, Cain/ Cabin and Rcan1.

Calcineurin is a heterodimer made up of the two subunits: CnA and CnB that are noncovalently attached. There are three isoforms of CnA: CnAα, CnAβ and CnAγ while CnB has two isoforms: CnB1 and CnB2. CnAγ and CnB2 are primarily found in the testis while the other isoforms are broadly distributed. CnA is an approximately 60 kDa protein and contains the catalytic domain, a binding domain for CnB, a calmodulin (CaM) binding domain and a autoinhibitory domain (23, 33, 41). The regulatory subunit CnB is approximately 19 kDa with four Ca^{2+} binding EF hands, two of high affinity and two with lower affinity. Complete activation of calcineurin involves binding of Ca^{2+} to the low affinity sites on CnB, followed by binding of Ca^{2+}/CaM to CnA (28). This leads to a conformational change where the autoinhibitory domain moves out of the catalytic groove. Activation occurs in the range of Ca^{2+} concentrations from 100 nM to several μM, but is also dependent on calmodulin and Mg^{2+} concentration.

A major target of calcineurin is the NFAT family of transcription factors (39). Of its five members (NFAT1-5), four (also known as NFATc1-c4) are regulated by calcineurin. They originated with the evolution of vertebrates, with a structurally novel combination of a DNA binding domain related to the Rel family of transcription factors, in addition to the amino terminal, Cn-regulated domain (54). The regulatory domain possesses 13 phosphorylation sites that are dephosphorylated by Cn. Dephosphorylation exposes a nuclear localization signal (NLS) which allows NFATs to move into the nucleus. The Rel-like domain alone binds only weakly to DNA, but stronger binding

occurs in cooperation with other factors such as AP1, GATA and MEF2 (31, 54).

Initially identified in T cells, NFAT signaling connects to the T cell antigen receptor (TCR) allowing expression of the IL-2 gene. With the advent of NFAT gene deletion, it has been discovered that NFATs also play a role in the development of the nervous system, heart, lungs, skeletal muscle, blood vessels and pancreatic islets, along with postnatal cardiac hypertrophy (54). NFAT signa ling is terminated once the protein is phosphorylated by dual specificity tyrosine kinase 1 (Dyrk1) or PKA, with the additional phosphorylation by GSK-3. This action causes leads to rapid export out of the nucleus and is thought to be a mechanism whereby brief Ca^{2+} signals alter gene expression. The majority of individual genes activated by NFATs are proinflammatory or proangiogenic. However, a few other target proteins have an even more diverse range of functions. One such strongly activated gene is Rcan1, also known as Dscr1 or Mcip1, so designated because it is present in the Downs syndrome critical region of chromosome 21. Rcan1 strongly inhibits the interaction between CN to NFAT and thereby the enzymatic activity of CN. CN action depends on docking target proteins through a PxIxIT motif, a recurrent domain originally identified in NFATs, but now also recognized in other transcription factors. Rcan1 also plays a role in mitochondrial function to maintain function and may promote ROS production (6, 37).

2. Actions of CCK mediated by Calcineurin in acinar cells

A Ca^{2+}/CaM dependent protein phosphatase has been demonstrated in mouse and rat pancreatic acinar cytosol (4, 10). Two dimensional gel electrophoresis of the P^{32} labeled proteins showed a limited set of targets dephosphorylated by CCK stimulation. One of these was a heat stable protein that had an approximate molecular mass of 24 kDa in which dephosphorylation was blocked by treatment with CsA (11). This protein, CRHSP-24, was sequenced, its phosphorylation sites were identified, and its crystal structure determined (11, 18, 27). It appears to be an RNA binding protein that participates in the stress response, but its main significance to date is as a surrogate marker of calcineurin activation in acinar cells.

Another action of this pathway involves calcineurin activation, NFAT dephosphorylation and pancreatic adaptive growth. Tashiro et al (48, 49) used the feeding of the trypsin inhibitor, camostat, to stimulate CCK mediated pancreatic adaptive growth without other organs being affected. Both CsA and FK506 blocked this growth. The amount of Cn and its activity were increased by the elevated CCK. CsA and FK506 did not affect the increase in plasma CCK. Several other signal transduction pathways were also affected by calcineurin inhibitors. Gurda, Guo et al, (13) showed that CCK both in vitro and in vivo released by trypsin inhibitor feeding induced NFAT translocation to the nucleus of acinar cells that correlated with activation of a NFAT luciferase reporter. NFATc1, c2 and c3 were present and all translocated. The effect of CCK was blocked by the calcineurin inhibitors CsA, FK506 and the overexpression of CAIN. Utilizing the model of trypsin inhibitor feeding to induce pancreatic growth, 39 genes were identified as having their expression increased in a manner blocked by FK506 (12). Many of these genes were predicted to be NFAT regulated and ChiP assay showed binding of NFATc1 induced by CCK. The most highly induced genes were Rcan1, FGF21, Kel, and SOCS3. A mouse line with an acutely-induced overexpression of Rcan1 in acinar cells showed inhibition of calcineurin-NFAT signaling and abrogation of pancreatic growth. Thus, Rcan1 can be considered a physiological feedback inhibitor. Other feedback inhibitor proteins such as Rgs2 were also induced and may function as a brake to growth. Interestingly, Rcan1 has more recently been shown to have a multitude of others functions –

for instance, Rcan1 overexpression in aging has been shown to promote diabetes (38).

Other actions of CCK that may involve calcineurin are the synthesis and secretion of digestive enzymes. Calcineurin inhibitors, FK506 and CsA dose dependently inhibited the stimulation of amino acid incorporation into protein within isolated rat pancreatic acini (42). In this study, FK506 blocked the phosphorylation of 4EBP-1 and reduced the formation of the eIF4F complex but did not affect the phosphorylation of ribosomal protein S6 or eIF4E. On this front, more confirmation and more detailed mechanistic understanding are needed. The effect of calcineurin inhibitors on secretion of digestive enzymes has also been studied. Early work reported that CsA and FK506 partially reduced stimulated amylase release from rat pancreatic lobules and isolated acini (10, 17, 53), but the relative potency of the inhibitors differed and did not always follow their ability to inhibit Cn activity. The same study also showed that CsA at at 1 to 400 μM concentrations affected the mitochondrial permeability pores. Followup studies, however, have not seen effects of FK506 on secretion in rat or mouse acini (20) (Williams JA, unpublished data). Thus, it is unclear whether secretion of digestive enzymes requires calcineurin.

Similar studies of calcineurin NFAT signaling have been carried out in islet beta cells and showed that the CN-NFAT pathway plays a key role in development, adaptive growth and insulin synthesis (7, 9, 14, 26). Also of interest are the varied effects of FK506 on beta cells, because of the use of this immunosuppressive agent following pancreatic islet transplantation.

3. Calcineurin – NFAT signaling and pancreatic disease

Pancreatitis

Increased Ca^{2+} flux is critical to the initiation of pancreatitis and experimental studies have showed the importance of calcineurin in initiating pancreatitis induced by caerulein (20, 30, 44), bile acids (29, 32, 34, 40), or the radiocontrast media, iohexol (22, 36). In all models, NF-κB and zymogens were activated and these effects were blocked by FK506, CsA, calcineurin inhibitory peptide or by gene deletion of CnAβ. In some studies a NFAT reporter was introduced and shown to be activated (34). In most of the above pancreatitis studies it is not clear if immunosuppressant drugs are acting on acinar or immune cells, and both are likely affected. The effects of pancreatitis in NFATc3 deficient mice have also been studied and pancreatitis induced by infusion of the bile salt taurocholate and arginine into the pancreatic duct was reduced in severity (1).

Pancreatic Cancer

Three of the four Cn-regulated NFATs (NFATc1, c2 and c4) expressed in pancreatic acinar cells have also been explicitly shown to be involved in carcinogenesis, including aspects of neoplastic transformation, cancer progression and cancer-related responses such as tumor microenvironment and immunogenicity. Both NFATc1 and NFATc2 are overexpressed in pancreatic ductal adenocarcinoma (PDAC) and convey a highly malignant, aggressive phenotype (3, 24). As a corollary, the activated, nuclear NFATs were more frequent and more highly expressed in advanced pancreatic cancer (PDAC) versus pre-cancerous (PanIN) stage (3). In most if not all cases, NFATs work in cooperation with other transcription factors, which appears to be necessary to elicit high affinity binding of common/overlapping target promoters. In pancreatic cancer cell lines, NFATc1 and NFATc2 together with Elk-1 regulate expression of the pro-proliferative and often (though contextually dependent) oncogenic factor Myc (3). NFATc2 (25) and more recently NFATc1 (2) were shown to form stable complexes with signal transducer and activator of transcription-3 (STAT3), and were implicated in cell transformation and pancreatic carcinogenesis, particularly in the pro-oncogenic $KRAS^{G12D}$ background. Thru EGFR, Wnt, Sox9

and other effectors, NFAT-STAT complexes regulate gene networks at the intersection of inflammation and cancer, thereby promoting acinar-ductal metaplasia and PanIN formation, a form of epithelial-mesenchymal transition (2, 16) as well as a tumor-permissive microenvironment (51). In other contexts, NFATc2 in association with AP1 promote invasiveness of breast cancer cells via transcriptionally-driven cyclooxygenase-2 (COX2) activation, inducing prostaglandins and matrix metalloproteinases such as MMP-2(55); NFATc1/c2 and COX2-dependent MMP-2 induction has also been demonstrated in highly invasive pancreatic cancer cells, particularly in a late-stage, TFGβ signaling driven carcinogenesis.

Given the aggressiveness of PDAC and typically late presentation at an unresectable stage, treatment options beyond the current standard chemotherapy protocols like FOLFIRINOX, are urgently needed (15). The NFAT pathway contributes to multiple lines of drug resistance to current therapies, and thus is an attractive target for improved treatments. Cn inhibitors, like CsA and FK506, are natural candidates, but a combination of marked side effects (immunosuppression, neurotoxicity, nephrotoxicity and cardiotoxicity) and high doses needed to achieve therapeutic response limit their usefulness. Sulindac, an NSAID with previously documented antineoplastic effects in pancreatic cells, and its analogue Phospho-sulindac (P-S) were recently trialed (35), with tumors that showed high levels of NFATc1 expression also showing decreased apoptosis induced by P-S. In pre-clinical trials, using CsA to inhibit NFATc1 expression prior to P-S administration re-sensitized pancreatic cancer cells and reduced tumor burden. Another option could be zoledronic acid (ZOL), FDA approved therapy for osteoporosis, successfully used as adjuvant therapy in breast and pancreatic cancer. Mechanistically, including in the pancreas, ZOL indirectly blocks GSK-3β activity and thereby impairs normal NFAT translocation/turnover, ultimately causing growth suppression (46). ZOL may also augment pancreatic cancer radiosensitivity (8). An attractive, low-risk approach may be the use of Aspirin, which inhibits the key NFAT effector COX-2 that has been well-documented to decrease both COX-2 expression and reduce risk of colorectal carcinoma (CRC) and pre-malignant adenomas (5). The usefulness of aspirin in more advanced stage CRC has been conflicting and it is possible the same will likely hold for pancreatic cancer, with utility demonstrated mostly as a preventative measure (47, 56). There is now widespread excitement about cancer immunotherapy and a subset of PDACs with high tumor infiltrating lymphocytes have also been shown to have high expression of PD-1/PD-L1 (57), a surrogate marker for responsiveness to immunotherapies such as Pembrolizumab (Keytruda). The interplay between immuno-oncology drugs and NFATs are only beginning to be investigated, particularly in relation to compensatory processes like T-cell exhaustion (19). The biology of these interactions will no doubt be complicated.

As presented in several research articles and reviews, future basic/translational cancer research will proceed in several directions simultaneously: early detection and risk assessment, molecular genomics for personalized, targeted therapy and improved prognostication, as well as efforts toward new drug development. In terms of clinical imaging and early detection, research-based efforts to develop antibody-tagged molecular probes are progressing (52), but to date none seem to have been specifically focused on Cn-NFAT pathway. Nonetheless, given strong overexpression of NFATs and subsequent multiplicative induction of their transcriptional effectors (i.e. Rcan), members of the pathway could be considered good targets, particularly as PDACs often show only rare cancer cells in a sea of desmoplastic stroma. Likewise NFATs themselves are only rarely mutated in pancreatic cancer (COSMIC database, accessed 05/15/2018), but pathways upstream, parallel or

otherwise directly tied to Cn-NFAT, such as Trp/Orai calcium channels, MAPK and JAK-STAT are frequently mutated. Thus, it will be important to examine if efforts such as targeted KRAS G12C specific inhibitor (21), or therapies aimed at specific upstream calcium channels (43) might have unintended consequences for Cn-NFAT axis. Since NFATs act as transcription factors and integrators of multiple intracellular signaling pathways, gene expression profiling (GEP) might also hold some potential. GEP has led to identification of putative NFAT signature in colorectal cancer associated with higher metastatic capacity (50), and similar efforts have led to sub-classification and improved prognostication in breast cancer. However, for clinical and technical reasons, parallel efforts in pancreatic cancer may be more challenging. Lastly, current pharmacological inhibitors of Cn-NFAT signaling do not suitably target the oncogenic functions of NFAT, failing to discriminate between NFAT and other targets of CN, specific NFAT complexes, or target cancer cells (24). Specific therapeutic approaches to target cancers in a highly targeted fashion are underway, particularly with peptide mimickers of endogenous Cn-NFAT pathway inhibitors, though clinical applications are still lacking (45).

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