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NFATC1: An Integrating Signal at the Heart of the Valve

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Establishment of functional tissues requires the proliferation of individual pluripotent cells followed by their differentiation to specialized ones. This process involves complex interaction of signaling molecules from outside and inside the cell, during which multiple genes will be over or under expressed [1]. Signaling starts either at the cell membrane level or intra-cellularly by the binding of various proteins to their corresponding receptors. Following this, activated downstream signals end up in the nucleus binding and activating target genes. The products of gene expression are proteins used for various functions including cell cycle regulation, proliferation, specialized function, adaptation to environment and apoptosis. Hence, genes expression plays a key role in all cellular processes starting from cellular maturation to death. An example of such a tightly regulated cascade of cellular events that affect organ structure and function is the Calcineurin/NFAT pathway that we will focus on in this editorial.

Nuclear Factor of Activated T-cells (NFATC) proteins are DNA-binding transcription factors that are evolutionary related to the Rel/NF-kB family [2]. This latter, is characterized by a highly conserved domain, referred to as RHF (Rel homology domain) that is used for binding to DNA. Five members (*NFATC1* to 5) exist in mammals, all located on different chromosomes with different pattern of expression and function. In addition, all NFAT genes are post-transcriptionally regulated by alternative splicing generating multiple isoforms [3]. The human *NFATC1* gene, the founding member of this family, is located on the long arm of chromosome 18 (q23) spanning approximately 130 kbp. Multiple proteins are generated by alternative splicing of the 10 exons that make up the gene: the biggest protein size being 930 amino acids.

Three different steps of activation have been defined for NFAT proteins: dephosphorylation, nuclear translocation, and DNA binding. In resting cells, NFAT proteins are phosphorylated and found exclusively in the cytoplasm [4]. Stimuli that trigger calcium mobilization result in rapid dephosphorylation of NFAT proteins by calcineurin. Calcineurin is a calcium-calmodulin dependent protein phosphatase formed by heterodimerization of two subunits A and B [5]. Subunit A is catalytic and binds calmodulin, whereas subunit B which possesses four EF-hands, is regulatory and it binds calcium with high affinity. Calcineurin dephosphorylates the Ca^{2+} calcineurin-sensitive subunits of NFATC, which are Serine-Rich Region (SRR) and Serine-Proline (SP) repeats at the N terminus of the protein4. Besides the RHD, this N-terminal region is highly conserved among all members of the family except *NFATC5* [4,6,7]. When NFAT becomes dephosphorylated, it undergoes conformational changes exposing previously inaccessible nuclear localization sequence and the DNA-binding region of NFAT. The dephosphorylated proteins show increased affinity for DNA and binds specifically to the consensus (A/T) GGAAA sequence [8].

Calcineurin is not only responsible for NFATC dephosphorylation in the cytosol, but its presence in the nucleus is also significant in ensuring the full transcriptional activity of NFATC proteins [9]. Calcineurin also appears to play a role in preventing the export of NFAT from the nucleus. The nuclear half-life of the NFAT protein alone is very short. In the absence of active calcineurin, it is rapidly transported back into the cytoplasm within minutes [10]. It will be subject to vain cycling across the nuclear envelope. The activation ends when intracellular free Ca^{2+} decreases and calcineurin dissociates from the transcription factor, export prevails, thereby ceasing the T-cell receptor signal.

Binding sites for NFAT proteins are present in the promoter/enhancer regions of several inducible genes, including the ones encoding cytokines, cell surface proteins, cyclin-dependent kinases, and cyclooxygenase-2 [2,4,11]. NFATC proteins are however weak transactivators by themselves; their transcriptional potency is boosted through their interactions with different classes of partner proteins that include the AP-1 family members, c-Fos and Jun, the MADS family, and the GATA zinc finger proteins [12-14]. These partners enhance NFAT transcriptional activity either by stabilizing its binding to DNA or by forming stable ternary complexes with common co-activators. For instance, co-operation with AP-1 proteins results in stabilization of the NFAT-DNA interaction. On the other hand, calcineurin-activated NFAT proteins functionally and physically interact with the GATA zinc finger transcription factors without affecting each other's DNA binding activities.

Most of the early work on NFAT has been related to immune cell activation and its mediators, such as cytokines. However, accumulating evidence has been demonstrating that NFAT transcription factors are present in a wide range of cell types and tissues including osteoctyes, endothelial vascular cells, myocytes, pancreatic and adipose tissue, etc. [4]. They play an important role in the control of gene expression during cell activation and differentiation, cell cycle progression, angiogenesis, and possibly tumorogenesis [15]. In fact, one of the best examples of how research focus shifted from the immune system to the cardiovascular system came from the inactivation of the *NFATC1* gene in mice, which led unexpectedly to an embryonic lethality due to a valvular defect in the heart. The mice have a defective development of the aortic and pulmonary valves with subsequent death at embryonic day 14-15 due to congestive heart failure. The process of valve formation is stopped just after the epithelial to mesenchymal transformation occurred suggesting a role for *NFATC1* in cell proliferation and maturation. This process is subject to tight regulation by growth factors mainly the Vascular Endothelial Growth Factor (VEGF) [16].

Our area of research on *NFATC1* is based on the mouse model generated by gene inactivation of the gene that leads to malformation of the valves suggesting that in humans any mutation in this gene could lead to the same phenotype. For that purpose, subjects from the

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Congenital Heart disease Genetic Program at the American University of Beirut Medical Center were enrolled in the study. Exons 1 to 8 which encode the coding sequence of *NFATC1* were sequenced.

Results showed several SNPs in different exons of various patients, except in exon 1 where no SNPs were found. Some of the polymorphisms were silent resulting in no change in the amino acid, whereas others did. Significance of the latter was further assessed using functional studies. In one of the subjects, PCR amplification of the exon 7 region yielded 2 amplicons, one at the expected size of the band, the other slightly of a larger size [17]. We first sequenced the lower band, and it turned out to be the right one. Sequencing results of the upper band showed that it corresponds to the same exon 7 but with an additional region. It is made up of 44 nucleotides (nt) in the intronic region, 56 nt downstream of the exon 3' boundary. The additional region is made of stretch of nucleotides that is a repeat of a duplicate already present. Moreover, 6 SNPs were found in the additional repeat. Checking for this allele frequency in our population supported a linkage to the Ventricular Septal Defect (VSD) phenotype but no association with valvular defects. The number of patients within the latter category were however very small. Two subsequent studies in the Chinese population showed opposing results with regards to this association. Further studies are needed to come up with a global conclusion and more importantly functional studies concerning the potential creation of an alternatively splice site at this position should be conducted.

Further genetic screening for our population of patients with valve defects did reveal the simultaneous presence of two SNPs in a patient with Tricuspid Atresia (AbdulSater et al, PLOS1, under press). The two heterozygous SNPs are in exon 2 and 8 leading to a P66L and I701L substitutions in the protein. These mutations were predicted to lead to the formation of beta sheet at position 66 and the removal of one at the position 702 in comparison with the wild type. These SNPs are novel and maybe disease causing since they were not found while screening 100 healthy controls.

Further investigation of the protein function was carried on. Transfection with the generated plasmid showed that both the wild and the mutant *NFATC1* proteins are located in the cytoplasm. Upon co-transfection with activated form of calcineurin, the double mutant protein failed to translocate to the nucleus in more than 80% of the cells while the wild type protein was exclusively found in the nucleus. Interestingly, the mutated protein was trapped on the nuclear membrane and was not present all over the cytoplasm as is the case in the absence of calcineurin. Functional studies showed that the mutated protein DNA binding affinity was reduced by 30% as compared to the wild type, and the transcriptional activation of downstream target genes is concomitantly partially inhibited though not completely. Finally, the interaction of the mutant *NFATC1* with GATA5 and HAND2 proteins were studied. Previous results produced by our group confirm such interaction with the wild type. *NFATC1* and GATA5 interact to synergistically activate downstream target genes like VEGF. This synergy was however dramatically abrogated by the mutations. All together, the results do point out to a major defect in the protein besides being unable to cross the nuclear membrane.

These results opened the way for a large screen of patients for possible *NFATC1* mutations. Our preliminary results do show additional mutations that might be disease causing also but that affect other aspects of heart development besides the valves. Functional studies are underway and ultimately knock-in mouse models will be the best way to prove that they are disease-causing.

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