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Down Syndrome and Vascular Disease: DSCR1 and NFAT Signaling

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1. Introduction

Down Syndrome (DS) is one of the most common genetic disorders to date, occurring in 1 out of every 800-1000 live births (Egan *et al.* 2004; Roizen & Patterson, 2003; Stoll *et al.* 1990). DS patients often display several developmental and cognitive deficiencies. Common phenotypes of DS patients include congenital heart disease, dysmorphic physical features, and early-onset Alzheimer's disease (AD). Since the discovery in 1959 that DS occurs from an extra copy of human chromosome 21 (hChr21) (Lejeune J, Gautier & Turpin, 1959), questions arose whether a 1.5-fold increase in a gene or set of genes were responsible for the phenotypes associated with DS. Sequence analysis of hChr21 identified over 225 genes and/or predicted genes (Hattori *et al.* 2000). With the recent advances in sequencing, the gene content of hChr21 is now estimated to exceed 300 genes (Roizen & Patterson, 2003). While typically characterized by complete trisomy of hChr21, several DS cases, however, demonstrate that partial trisomy of hChr21 is enough to elicit the phenotypes associated with DS (Stoll *et al.* 1990), hence arising the concept of a Down Syndrome Critical Region (DSCR). The DSCR theory suggests that enhanced expression of a few genes located in this critical chromosomal region (between markers D21S17 and D21S55 (Delabar *et al.* 1993; Korenberg *et al.* 1994)) are responsible for some, if not all, of the features of DS. Olson LE *et al.* show, however, that triplication of this region alone is not enough to fully manifest the phenotypes associated with DS (Olson *et al.* 2004; Olson *et al.* 2007). These findings contradict the longstanding DSCR theory, indicating that the genetic instability seen with hChr21 triplication is not merely from excess gene expression. Rather, the functional interactions occurring within a set of overexpressed genes may explain the various phenotypes observed in DS.

Many of the triplicate genes on hChr21 are implicated in several diseases. Cohorts of genes, interestingly, have been associated with pathologies that appear to be more common for DS individuals. A group of at least 16 genes on hChr21 have been correlated with a role in energy and reactive oxygen species (ROS) metabolism (Roizen & Patterson, 2003). Several studies have linked mitochondrial dysfunction and metabolic disorders with DS (Arbuzova, Hutchin & Cuckle, 2002). *In vitro*, DS neurons were observed to have 3- to 4-fold increases in reactive oxygen species compared to control cells, with elevated rates of consequent cell apoptosis (Busciglio & Yankner, 1995). Degeneration of DS neurons was rescued by administration of free-radical scavengers, suggesting DS neurons are unable to efficiently metabolize ROS. DS patients are also evidenced to have increased mitochondrial superoxide

production compared to control groups (Capone *et al.* 2002). In addition, Busciglio J *et al.* show altered processing of the amyloid precursor protein (APP) in DS astrocytes and neurons (Busciglio *et al.* 2002). Elevated levels of APP, a gene also located on hChr21, have been associated with AD progression. Given that DS patients invariably display the characteristic plaques and tangles suggestive of AD (Hardy & Selkoe, 2002), this 1.5-fold increase in particular hChr21-located genes may explain the metabolic-related diseases associated with DS patients.

Another cohort of at least 10 genes located on hChr21 have been demonstrated to influence the central nervous system (CNS), and may hence play a role in the neuropathogenesis of DS (Capone, 2001). Considering the major phenotypes of DS patients include altered brain development, neuronal loss, and Alzheimer's-like neuropathology, the identification of CNS-related genes is not surprising. As demonstrated by these two gene clusters, phenotypic outcome is dependent not only on genotype but also on successive protein interactions. It is therefore possible that additional sets of genes, when in excess as a result of trisomy hChr21, lead to the manifestation of other DS-related pathologies, such as vascular-related pathologies.

2. Clinical observations and chapter overview

While developmental and behavioral disorders have been extensively studied in adults with DS, relatively little is known regarding their propensity to vascular-related diseases. Several intriguing clinical observations relating to the vasculature have been reported amongst DS patients over the last several decades. Unlike the aforementioned developmental deficits of DS individuals, DS patients appear to have a unique advantage in the context of several vascular pathologies.

2.1 Cancer incidence in DS patients

While adults with DS have been shown to have a higher incidence of leukemia compared to the general public, the risk of solid tumors appear to be strikingly low in individuals with DS (Hasle, Clemmensen & Mikkelsen, 2000). A population-based study on causes of mortality in over 17,000 DS patients showed a significant lack of malignant neoplasms as a cause of death (Yang, Rasmussen & Friedman, 2002). These findings suggest that individuals with DS express possible tumor-suppressor genes on hChr21, such that trisomy of a set of genes results in an antineoplastic effect on solid tumors. It is possible that these set of genes modulate a common biological mechanism that is critical for tumorigenesis and/or cancer progression.

2.2 Atherosclerosis in DS patients

A post-mortem, observational study published in 1977 found that 40-66 year old patients with DS displayed an almost complete absence of atherosclerotic plaque formation in arteries, compared to those without DS (Murdoch *et al.* 1977). Additional population studies in DS patients have found consistent results (Draheim, Geijer & Dengel, 2010; Yla-Herttuala *et al.* 1989), leading one to speculate that an unidentified factor(s) specific to DS patients explains the near absence of atherosclerotic plaque formation. Considering the vascular similarities between tumorigenesis and atherosclerosis, it is possible these events are driven by a shared molecular mechanism.

2.3 Other vascular pathologies in DS patients

Studies focusing on vascular tone show that DS patients have extremely low percentages of hypertension (Kerins *et al.* 2008). Significant decreases in both systolic and diastolic pressures were observed in DS patients when compared to reference populations (Morrison *et al.* 1996; Richards & Enver, 1979). Low blood pressures have also been observed in Alzheimer's disease (AD) (Burke *et al.* 1994; Landin *et al.* 1993), suggesting the mechanisms driving AD contribute to the observed hypotensive tendencies of DS patients. The already mentioned propensity for DS patients to develop AD, in addition to blood pressure levels similar to that of AD patients, strongly suggests a shared genetic contribution(s) of hChr21-located genes.

The observed decrease in solid tumor growth, lack of atherosclerotic plaque formation, and hypotensive nature of DS adults indicates onset of these vascular disorders may share an underlying molecular mechanism. This chapter will aim to discuss the observed differences in the vasculature of DS patients based on current literature to date. As of now, there is no concrete evidence distinguishing DS patients from non-DS patients on a genetic level concerning vascular anomalies. Correlative studies, however, suggest DS individuals harness a genetic advantage in regard to vascular health and disease.

3. Tumorigenesis in Down Syndrome

The molecular mechanisms underlying oncogenesis are undoubtedly one of the most intricate processes to date. Accumulation of multiple genomic mutations leads to the dysregulation of cell signaling pathways that are critical for controlling basic cellular functions including cell growth, survival, and cell fate. The extensive cross-talk between various signaling cascades highlights the sophisticated communication that transpires during cancer development and progression.

3.1 Calcium/calcineurin/NFAT signaling

Calcium (Ca^{2+}) fluxes are involved in multiple cell processes such as cell metabolism, membrane transport, cell permeability, and apoptosis. Changes in intracellular Ca^{2+} levels affect downstream gene expression and cell function through various mechanisms. In particular, intracellular Ca^{2+} concentrations often regulate kinase and phosphatase activity through the Ca^{2+} -binding protein, calmodulin (CaM). Increases in intracellular Ca^{2+} have been demonstrated in various cell types to activate the Ca^{2+} /CaM-dependent serine-threonine protein phosphatase, calcineurin (Cn). The Cn pathway is critical for the transduction of many extracellular, adaptive stimuli. Activated Cn subsequently dephosphorylates target proteins, including the nuclear factor of activated T-cells (NFATc1-c4) family of transcription factors (**Figure 1**). The NFAT family was originally identified in lymphocytes for its role in cytokine gene expression. While first identified in immune cells, recent findings show NFAT proteins are ubiquitously expressed. NFAT at the basal state are localized to the cytoplasm in an inactive form, with more than 20 identified phosphorylation sites (Okamura *et al.* 2000). CaM/Cn-mediated dephosphorylation of NFAT promotes nuclear translocation and target gene transcription (**Figure 1**). As downstream effectors of the Cn signaling pathway, NFAT-dependent gene transcription influences a range of cellular functions, including endothelial cell migration (Minami *et al.* 2004), skeletal muscle differentiation (Rothermel *et al.* 2000), cardiac valve development (Lange, Molkentin & Yutzey, 2004), and vascular smooth muscle cell adaptation (Crabtree & Olson, 2002; Horsley & Pavlath, 2002; Lee *et al.* 2010).

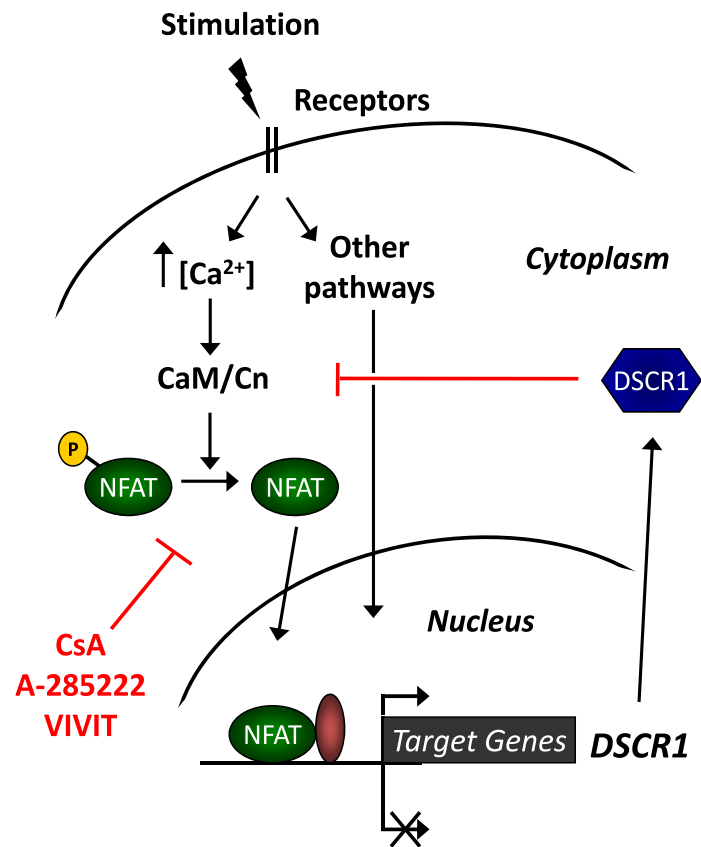


Fig. 1. The Cn/NFAT signaling pathway. calmodulin (CaM), calcineurin (Cn), cyclosporin A (CsA) (adapted from Viola, J.B.P., 2005)

Given the versatility of NFAT activation, it is not surprising that solid tumors and haematological malignancies are correlated with overexpression of NFATc isoforms. For example, pancreatic carcinomas display elevated levels of NFATc1 for transcriptional activation of the c-myc oncogene (Buchholz *et al.* 2006). Studies using human breast carcinoma cell lines also show NFATc2 expression promotes carcinoma migration and invasion (Jauliac *et al.* 2002). The increased NFATc activity in malignant cells suggests activation of downstream NFATc targets is critical for tumor progression. Cyclooxygenase-2 (COX2/PTGS2) has been reported by several groups as an NFAT-dependent gene in non-lymphoid tissues, including endothelial cells (Hernandez *et al.* 2001), mesangial cells (Sugimoto *et al.* 2001), and vascular smooth muscle cells (Pritchard, Jr. *et al.* 1994). As an enzyme involved in prostaglandin formation, COX2 expression plays an important role in cell survival and angiogenesis. A gain-of-function approach shows that forced overexpression of COX2 alone is enough to induce mammary gland tumorigenesis (Liu *et al.* 2001). On the converse, inhibition of COX2 significantly reduces colon tumor incidence and multiplicity in a dose-dependent manner (Reddy *et al.* 2000). These findings strongly support the notion that tumor growth/invasion necessitates an abundance of COX2 levels. The increased expression of COX2 in a multitude of cancer lines (*i.e.* gastric, esophageal, breast, bladder, cervical, colorectal, endometrial (Brown & DuBois, 2005; Duque, Fresno & Iniguez, 2005b)) have thus launched the production of several COX2 inhibitors with therapeutic potential. It is, however, important to note the importance of COX2 in other cell types. Unlike the constitutively expressed COX1 isoform, COX2 expression occurs in a

limited number of cell types, primarily in response to cytokines, inflammatory mediators, and tissue damage (Lipsky *et al.* 2000). The inducible nature of COX2 activation results in prostaglandin formation, including prostacyclin, a vasodilator and a potent inhibitor of platelet aggregation. Prostacyclin formation in vascular endothelial cells thus provides a mechanism by which the thrombotic response in platelet activation can be regulated (Rudic *et al.* 2005). Hence, COX2 expression is necessary to maintain vascular health. While COX2-selective inhibitors may attenuate tumor angiogenesis, COX2 antagonists will undoubtedly shift eicosanoid balance toward a prothrombotic state, promoting the risk for cardiovascular events (Mukherjee, Nissen & Topol, 2001). Targeting other proteins involved in Cn/NFAT signaling aside from COX2 may yield similar anti-neoplastic results.

Tumor survival and growth is highly-dependent on a vascular system for delivery of essential nutrients and oxygen. A tumor's vasculature also provides a mechanism by which aberrant cells can disseminate and metastasize to other organs. Without a constant blood supply, tumor volumes tend to remain 1-2mm³ in size (Brown & DuBois, 2005). Angiogenesis is a multi-step process that involves migration, proliferation, and differentiation of both endothelial and vascular smooth muscle cells for generation of mature vessels. Tumor cells can facilitate an 'angiogenic switch' by altering gene transcription and the surrounding microenvironment. Somatic mutations of various proto-oncogenes and/or tumor suppressor genes offset the balance between activators and inhibitors of angiogenesis, ultimately favoring angiogenic activity (Hanahan & Weinberg, 2000). Pro-angiogenic gene expression is induced by a number of physiological stimuli, such as hypoxia. Cells in hypoxic conditions produce the hypoxia-inducible factor (HIF) transcription factor, where expression of HIF-1 results in Vascular Endothelial Growth Factor (VEGF) transcription (Forsythe *et al.* 1996). VEGF, a major stimulator of angiogenesis, is a potent endothelial mitogen involved in both physiological and pathological blood vessel formation. VEGF is critical for proper embryonic vasculogenesis, as even heterozygous VEGF-deficient mice display abnormal blood vessel formation and consequent embryonic lethality (Carmeliet *et al.* 1996). While necessary for proper blood vessel development, excess VEGF can result in pathological angiogenesis. HIF-1-mediated VEGF expression and activation of other angiogenesis-related genes exemplifies the adaptive responses seen in tumor survival and growth. VEGF has also been demonstrated to induce COX2 expression in a Cn/NFAT-dependent manner (Duque, Fresno & Iniguez, 2005c). As previously mentioned, COX2 expression is critical for tumor cell migration and tube formation. The emerging role of NFAT signaling in oncogenesis is further highlighted, as pharmacological inhibition of NFAT blocks VEGF-stimulated COX2 expression and subsequent cell proliferation and/or angiogenesis both *in vitro* and *in vivo* (Hernandez *et al.* 2001). These results imply activation of Cn/NFAT signaling is required for the formation of new blood vessels, where regulation of angiogenesis is dependent on a local balance between angiogenic factors and inhibitors. The identification of Cn/NFAT activators and downstream NFAT target genes in the last few decades has therefore helped elucidate the biological mechanisms underlying oncogenesis.

3.2 DSCR1 in cancer progression

Cancer incidence for all solid tumors and malignancies have been shown in multiple DS population studies to be significantly lower in DS patients compared to control groups (Hasle *et al.* 2000; Yang *et al.* 2002). Interestingly, a large focus has been placed on the role of the Down Syndrome Candidate Region 1 (DSCR1/RCAN1/MCIP1) gene in cancer

progression. Located on hChr21, DSCR1 has been demonstrated as a negative feedback regulator of Cn/NFAT signaling through physical binding to the catalytic subunit of Cn (Fuentes *et al.* 2000) (**Figure 1**). In endothelial cells, VEGF and thrombin stimulation dramatically upregulate DSCR1 expression in a Cn/NFAT dependent manner (Iizuka *et al.* 2004; Minami *et al.* 2004). Several studies in endothelial cells have shown that DSCR1 overexpression inhibits NFAT transcriptional activity, suppresses NFAT nuclear accumulation, and attenuates NFAT-dependent inflammatory marker gene expression, such as COX2 (Hesser *et al.* 2004; Yao & Duh, 2004). Studies also show DSCR1 overexpression in endothelial cells reduces vascular density in matrigel plugs *in vitro* and in tumor growth in mice (Minami *et al.* 2004). Lastly, functional data indicate constitutive expression of DSCR1 impairs endothelial cell proliferation and tube formation (Minami *et al.* 2004). Taken together, VEGF and thrombin-mediated endothelial cell proliferation/vascularization induces expression of DSCR1 as a mechanism to negatively regulate the angiogenic processes.

Researchers have attempted to explain the lack of tumorigenesis in the DS population through use of genetically modified mice. Comparative mapping between mice and humans identify that hChr21 is genetically homologous to mouse chromosome 16 (mChr16) (Reeves & Citron, 1994). Thus, mice exhibiting trisomy of mChr16 (Ts16) were originally developed to study DS. Ts16 mice, however, die *in utero* and therefore pose as a limitation. In addition, several genes located on mChr16 are found on human chromosomes other than hChr21. The observed embryonic lethality as well as gene dosage imbalances resulted in generation of the Ts65Dn mouse (Reeves *et al.* 1995). The Ts65Dn mouse model reflects partial trisomy of hChr21, where mice have been reported to display a variety of phenotypes, including behavioral abnormalities consistent with DS (Reeves *et al.* 1995). Ts65Dn mice are now widely used to study DS at all stages of development.

A recent publication highlights the suppression of tumor growth seen in DS individuals using two different models: 1) the Ts65Dn mouse and 2) a DSCR1-transgenic mouse. Ts65Dn mice possess extra genetic information including the DSCR1 gene - Ts65Dn mice exhibit a 1.7-fold increase in DSCR1 protein expression compared to diploid controls (Baek *et al.* 2009). Baek KH *et al* demonstrate that both lung carcinoma and melanoma cell growth was significantly suppressed in Ts65Dn mice, when compared to control groups (Baek *et al.* 2009). Under the transplantable tumor model, Ts65Dn mice also display significant decreases in tumor growth and microvessel density (Baek *et al.* 2009). Similar results were seen when using DSCR1-transgenic mice, suggesting that one extra copy of DSCR1 is sufficient for tumor growth suppression. Given that hChr21 contains over 200 genes, however, it is likely that other genes act in concert with DSCR1 to facilitate tumor suppressive effects. Another gene of interest includes Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase (DYRK1A), a nuclear serine/threonine kinase located on hChr21. DYRK1A phosphorylates several transcription factors including NFAT. DYRK1A can therefore reduce Cn/NFAT-dependent transcription through its ability to export nuclear NFAT. Baek KH *et al* also show that endothelial cells overexpressing DSCR1 together with DYRK1A demonstrate an even greater suppression of VEGF-mediated cell proliferation (Baek *et al.* 2009). Future work by Baek KH *et al* will focus on additional hChr21-located genes known to suppress tumor angiogenesis through alternate mechanisms (Ryeom, Baek & Zaslavsky, 2009), such as COLLAGEN XVIII (COL18A1), a precursor to the angiogenesis inhibitor endostatin, and ADAMTS1, a metalloproteinase known to regulate the anti-angiogenic function of thrombospondin-1 (Iruela-Arispe, Carpizo & Luque, 2003;

Zaslavsky *et al.* 2010). In summary, the tumorigenic protective effects seen in DS adults may be due to increased expression of select hChr21-located genes, including DSCR1.

4. Atherosclerosis in Down Syndrome

Atherosclerosis progression is a complex process involving several cell types, multiple biological mechanisms, and environmental factors. The pathogenesis of atherosclerotic plaque formation is highly dependent on hemodynamic flow patterns (DeBaakey, Lawrie & Glaeser, 1985; Vanderlaan, Reardon & Getz, 2004), thrombotic processes, and inflammatory signaling cascades (Lusis, 2000). Atherosclerosis is one of the leading causes of mortality in Westernized societies, and thus several cardiovascular risk factors have been identified over the last few decades in an effort to predict cardiovascular events. Given that atherosclerosis is characterized by accumulation of lipids in the large arteries, it is not surprising that elevated plasma triglyceride levels have been demonstrated as a direct propagator of cardiovascular disease (Austin, Hokanson & Edwards, 1998; Hokanson & Austin, 1996). Other plasma measurements indicative of cardiovascular risk include reduced levels of high-density lipoprotein (HDL) (Assmann *et al.* 1996; Gordon *et al.* 1977), and elevated serum levels of C-reactive protein (CRP), an indicator of inflammation (Mendall *et al.* 1996). High values for body mass index have also been demonstrated as a measure of cardiovascular risk (Berenson *et al.* 1998).

Atherosclerotic cardiovascular disease has been associated with metabolic syndrome, which includes several clinical disorders such as obesity, insulin resistance, glucose intolerance, hypertension, and dyslipidemia (Moller & Kaufman, 2005). Consistent with the aforementioned metabolic deficiencies associated with DS patients, individuals with DS also exhibit impaired lipid metabolism (Ishihara *et al.* 2009). In addition, DS patients have lower levels of low-density lipoprotein receptor (LDLR) expression compared to adults without DS (Corsi *et al.* 2005). Interestingly, lack of or dysfunctional LDLR expression results in familial hypercholesterolemia, a genetic disease characterized by elevated cholesterol levels and premature coronary artery disease (Brown & Goldstein, 1976). LDLR-null mice have since been generated and is now a widely used atherosclerotic mouse model (Ishibashi *et al.* 1993; Wouters *et al.* 2005). Lastly, DS patients exhibit significantly elevated levels of plasma triglycerides, low levels of HDL, high levels of CRP, and high levels of total body fat (Corsi *et al.* 2005; Draheim *et al.* 2010; Nishida *et al.* 1977). Taken together, these findings imply DS adults are surely at risk for cardiovascular events. DS patients, however, are significantly less susceptible to cardiovascular disease. A comparison of intimal-to-medial thickness (IMT) of the common carotid artery in DS adults show significantly lower IMT ratios when compared to control groups (Draheim *et al.* 2010). These findings corroborate the observational findings in 1977 where DS patients were proposed to be an “an atheroma-free model” (Murdoch *et al.* 1977). One can speculate that DS patients thus express an altered gene expression profile where an additional copy of hChr21-located genes results in protection against atheroma formation, despite the many validated plasma indicators of cardiovascular risk. Similar to the identified gene clusters responsible for the metabolic and developmental deficiencies in DS patients, it is highly likely that a cohort of genes located on hChr21 is responsible for the protective effects against cardiovascular disease. Identification of these genes will therefore provide a therapeutic direction as we continue to understand the etiology behind atherosclerotic plaque formation.

4.1 Vascular smooth muscle and atherogenesis

At the cellular level, atherosclerosis progression is characterized by lipid accumulation and fibrous lesion formation in large arteries, resulting in chronic arterial wall inflammation. The release of cytokines and growth factors induce vascular smooth muscle cells (SMC) to undergo a process known as phenotypic modulation, whereby an adult, contractile SMC alters its expression profile to facilitate cell proliferation, migration, and/or inflammatory cell recruitment (Shin *et al.* 2008) (**Figure 2**). Phenotypic modulation is a key event underlying atherogenesis and in-stent restenosis that involves several signaling cascades, including the Cn/NFAT pathway. Activation of Cn/NFAT signaling has been shown to induce vascular SMC proliferation and migration in response to receptor tyrosine kinase and G-protein-coupled receptor agonists, respectively (Liu, Dronadula & Rao, 2004; Yellaturu *et al.* 2002). Cn/NFAT activity is also critical for vessel wall assembly (Graef *et al.* 2001). Cyclosporin A (CsA) and the VIVIT peptide are commonly used Cn and NFAT inhibitors, respectively (**Figure 1**). A third pharmacological compound, A-285222, also inhibits Cn/NFAT signaling (Lee *et al.* 2010; Nilsson *et al.* 2007). Inhibition of Cn/NFAT signaling through use of either CsA or A-285222 attenuates Cn/NFAT-mediated SMC migration and proliferation *in vitro* (unpublished, **Figure 1**). Previous literature also shows that blocking Cn/NFAT signaling *in vivo* significantly inhibits vascular-injury-induced neointima formation (Liu *et al.* 2005), supporting the notion that Cn/NFAT signaling is intimately involved in the smooth muscle response to vascular injury. While NFAT-dependent gene regulation has been widely studied in lymphocytes, cardiac and skeletal muscle, the lack of known NFAT target genes in SMCs limits our understanding of Cn/NFAT signaling in vascular remodeling.

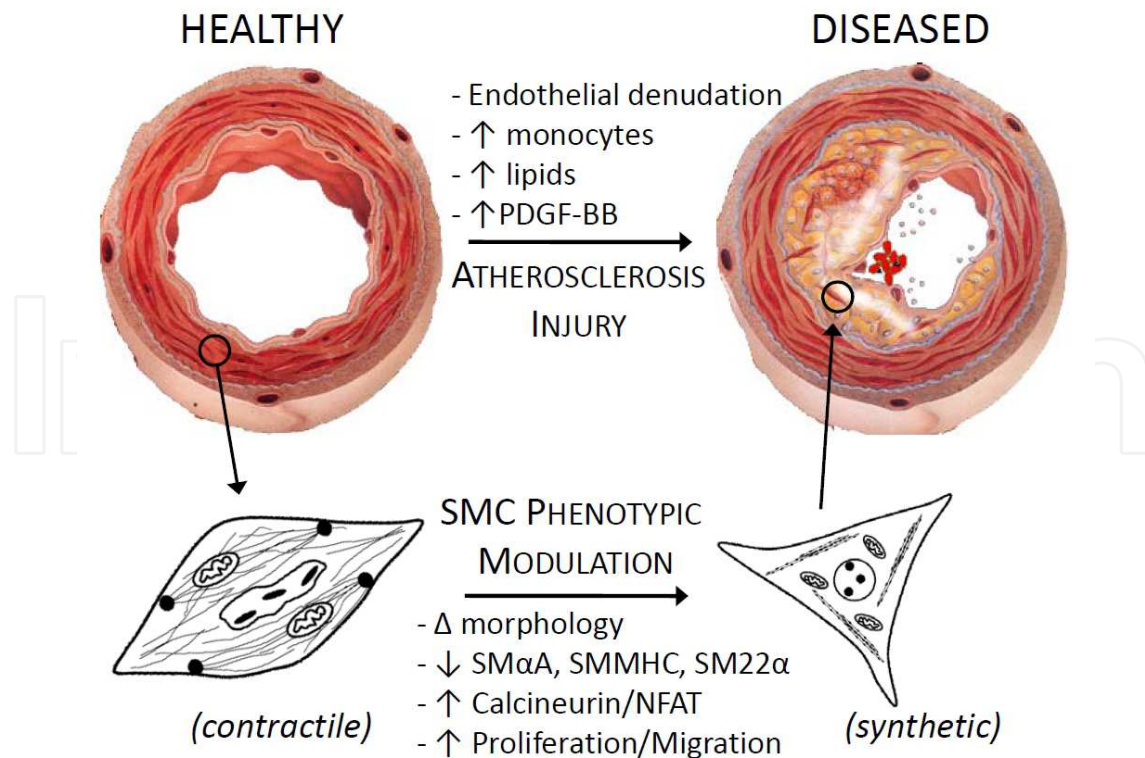


Fig. 2. The interplay between SMC phenotypic modulation and Cn/NFAT signaling in vascular disease.

4.2 Identification of DSCR1 using integrative genomics

We recently developed an unbiased, top-down, integrative genomics approach to determine downstream targets of Cn/NFAT activation in vascular SMCs (Lee *et al.* 2010). In brief, whole-genome expression data sets were analyzed to identify differentially upregulated genes in human, mouse, and rat SMCs. Comparison between control and phenotypic modulatory stimuli identified 63 species-conserved, upregulated genes. Differentially upregulated genes were then compared against an *in silico* NFAT-ome (a list of gene promoters containing at least one species-conserved physical NFAT binding site), yielding 18 potential Cn/NFAT-dependent genes. Use of pharmacological Cn/NFAT inhibitors substantiated the NFAT-dependent role for COX2, a previously identified Cn/NFAT dependent gene, and revealed the Cn/NFAT-dependent property of DSCR1 in vascular SMCs (Lee *et al.* 2010). DSCR1 has been reported in other cell types to function as a negative feedback regulator of Cn/NFAT signaling (**Figure 1**). Both gain- and loss-of-function studies were conducted to determine the role of DSCR1 in vascular SMCs. Overexpression of DSCR1 resulted in decreased NFAT transcriptional activity and reduced expression of other Cn/NFAT-dependent genes, such as COX2. Conversely, knockdown of endogenous DSCR1 enhanced NFAT transcriptional activity suggesting DSCR1 also functions as a negative regulator in vascular SMCs (Lee *et al.* 2010). Lastly, analysis of *in vivo* whole-genome expression arrays found significant DSCR1 upregulation with acute vascular injury in mouse carotid arteries, regardless of strain type (Lee *et al.* 2010). The significant induction of DSCR1 exemplifies its functional importance and may be critical for modulating the vascular injury response. Similarly, an extra copy of the DSCR1 gene in DS patients may function in an atheroprotective manner through its ability to negatively regulate Cn/NFAT signaling. The recent study highlighting significantly less tumor growth in DSCR1-transgenic mice (Baek *et al.* 2009) fosters the regulatory role of DSCR1 in Cn/NFAT-mediated cell growth. It is possible DSCR1 functions in both endothelial cells and vascular SMCs as a modulator of cell proliferation, thereby providing a shared mechanism underlying tumorigenesis and atheroma formation.

4.3 Ongoing research

Several studies are currently underway in an effort to understand the functional role of DSCR1 in SMC phenotypic modulation and atherosclerotic plaque formation. DSCR1-deficient mice have since been obtained and are proving to be instrumental as we continue to elucidate the role of DSCR1 in atherogenesis. DSCR1-related family members, however, have been identified in most eukaryotic organisms. In humans, DSCR1 family members include DSCR1-like 1 (DSCR1L1/ZAKI-4/RCAN2) and DSCR1-like 2 (DSCR1L2/RCAN3) (Strippoli *et al.* 2000). Interestingly, DSCR1L1 mimics the inhibitory effects of DSCR1 on Cn signaling in endothelial cells and inhibits angiogenesis (Gollogly, Ryeom & Yoon, 2007). In lymphocytes, DSCR1L2 blocks nuclear NFAT translocation and inhibits NFAT-dependent cytokine gene expression (Mulero *et al.* 2007). Given the functional overlap between the DSCR1 family members, global DSCR1^{-/-} mouse models may possibly exhibit compensation by DSCR1L1 and/or DSCR1L2. In addition, DSCR1 plays a critical role in each of the major cell types seen with atherogenesis and will therefore be difficult to attribute our findings as a vascular SMC-specific response. Until a SMC-specific conditional knock-out mouse study is carried out, a conservative approach must be taken when interpreting results gathered from DSCR1-null mice given the complexities surrounding

DSCR1 expression and regulation in other cell types. Nonetheless, the data gathered will provide a better understanding of DSCR1 function.

Apolipoprotein E (ApoE) is critical for lipoprotein metabolism. Defects in ApoE expression can consequently increase plasma cholesterol and triglyceride levels. Hence, targeted mutagenesis of the ApoE gene in mice promotes elevated serum cholesterol levels, where combination with a high fat/cholesterol diet results in massive accumulation of cholesterol and causative atherosclerotic lesion formation. DSCR1^{-/-}; ApoE^{-/-}-double knockout mice have been generated in an effort to study how the absence of DSCR1 affects atherosclerotic plaque formation. These double-null mice have been placed on a 20-week, high fat/cholesterol diet to ensure plaque formation, where atherosclerotic plaque size will be quantified and compared against proper control groups. Given the literature to date, we predict atherosclerotic lesion formation and size to significantly increase in DSCR1^{-/-}; ApoE^{-/-} double-null mice. The absence of DSCR1, a negative regulator of Cn/NFAT activity, should in theory promote SMC proliferation and infiltration into the neointima. A recent publication, however, emphasizes the dual role of DSCR1, suggesting DSCR1 is necessary for both Cn/NFAT activation and self-regulation (Liu, Busby & Molkentin, 2009). This effect has been demonstrated in cardiomyocytes and hypertrophy, an adaptive response driven by Cn and NFATc4 signaling (De Windt *et al.* 2001; Molkentin *et al.* 1998). Several groups have shown an attenuation of cardiac hypertrophy with overexpression of DSCR1 (Rothermel *et al.* 2001). DSCR1^{-/-}; DSCR1L1^{-/-} double-null mice were thus hypothesized to demonstrate significantly greater cardiac hypertrophy. DSCR1^{-/-}; DSCR1L1^{-/-} double-null mice, however, exhibit the reverse response – cardiac hypertrophy is impaired in response to adrenergic stimulation or exercise, similar to responses seen in calcineurin A β ^{-/-} mice (Sanna *et al.* 2006). These results suggest a basal level of DSCR1 is necessary to properly activate Cn/NFAT signaling. The DSCR1 yeast homolog, RCN1/CBP1, has also been functionally characterized as a facilitator of Cn/NFAT signaling (Kingsbury & Cunningham, 2000). The notion that DSCR1 may also activate Cn/NFAT signaling highlights the elaborate nature of this protein, suggesting that the paradoxical function of DSCR1 depends on initial protein concentrations (Shin *et al.* 2006). It is thus possible we may instead observe a decrease in atherosclerotic lesion formation in the DSCR1^{-/-}; ApoE^{-/-} double-null mice. With this in mind, we have also placed DSCR1^{+/-}; ApoE^{-/-} mice to observe how DSCR1 haploinsufficiency affects plaque formation. The presence of one DSCR1 allele will allow for minimal DSCR1 expression, yet also test the effects of reduced DSCR1 expression on lesion formation.

We are also performing several *in vitro* assays on primary aortic SMC cultures from DSCR1^{-/-} mice to determine the consequences of DSCR1 absence on cell functionality. With our recent findings demonstrating DSCR1 as a negative regulator of Cn/NFAT signaling in vascular SMCs (Lee *et al.* 2010), one would hypothesize that DSCR1-null vascular SMCs to exhibit increased cell proliferation in response to phenotypic modulatory stimuli. Preliminary data, however, show a reduced proliferative response in DSCR1-null vascular SMCs when stimulated with either platelet-derived growth factor (PDGF-BB) or serum (unpublished). These initial results support the emerging dual role of DSCR1, suggesting basal levels of DSCR1 is necessary for Cn/NFAT activation. While our model cannot fully explain the almost complete absence of atheroma formation in DS patients, our results will not only help elucidate the functional role of DSCR1 in atherogenesis, but also provide insight on the effects of trisomy 21 through a single gene approach.

5. Vascular tone and Down Syndrome

Enzyme activity of endothelial nitric oxide synthase (eNOS) plays a critical role in determining vascular tone through its ability to generate nitric oxide (NO) and consequently affect vascular smooth muscle contraction. Regulation of eNOS enzymatic activity is a highly dynamic system involving several kinases and phosphatases with significant downstream effects on NO-signaling (Duran, Breslin & Sanchez, 2010). While phosphorylation of eNOS is typically associated with enzyme activation, several groups demonstrate that dephosphorylation of eNOS can also trigger eNOS activation and subsequent NO production (Harris *et al.* 2001; Kou, Greif & Michel, 2002; Thomas, Chen & Keaney, Jr., 2002). The Cn phosphatase has been shown to target several proteins aside from NFAT, including eNOS (Kou *et al.* 2002). While the absolute role of DSCR1 is unknown in the vasculature, recent literature suggests DSCR1 plays a vital role in vessel contraction. DSCR1^{-/-} mice have been reported to exhibit an attenuated vasoconstriction response with phenylephrine treatment compared to appropriate controls (Riper *et al.* 2008). Riper DV *et al* speculate that the altered vascular constriction in DSCR1^{-/-} mice may be due to Cn dysregulation, thereby facilitating excess endothelial NO production. Based on the proposed mechanism, overexpression of DSCR1 should then limit NO formation and promote vasoconstriction. As stated earlier, however, adults with DS are significantly hypotensive when compared to control groups (Morrison *et al.* 1996; Richards & Enver, 1979). Although the attenuated vasoconstriction response in DSCR1^{-/-} mice may contradict the hypertensive nature of DS patients, the account by Riper DV *et al* is the first publication to date revealing a potential role of DSCR1 in vascular tone.

It is also important to note that these observations of a weakened vasoconstriction response in DSCR1^{-/-} mice reflect those of mesenteric rather than carotid arteries. While the underlying effects of NO on smooth muscle and vascular tone may be synonymous between the differing vascular beds, vasoconstriction could be regulated through different mechanisms. The effect of vascular bed type is further exemplified by the fact that while DS patients may be significantly hypotensive, they are also at an increased risk of developing pulmonary arterial hypertension (PAH) (Cua *et al.* 2007). The observed increase in PAH incidence amongst DS individuals may be due to several factors such as chronic upper airway obstruction (Jacobs, Gray & Todd, 1996) and abnormal pulmonary vasculature growth (Chi, 1975). Vascular tone therefore appears to depend not only on the vascular bed of interest but also on peripheral influences. In addition, basal levels of DSCR1 may be required for proper Cn/NFAT function, as previously described. Future contractility studies in haploinsufficient DSCR1^{+/-} mice may thus provide clarity on the true role of DSCR1 in vascular tone. This study clearly demonstrates the widespread effect of DSCR1 loss, where dysregulation of Cn/NFAT activity in the endothelium alters paracrine signaling molecule production, creating invariable effects on neighboring cell types. The prevalent nature of DSCR1 prompts consideration for cell-cell interaction effects as we continue to decipher DSCR1 function in vascular disease.

6. NFAT regulation and other considerations

The importance of calcium signaling, NFATc transcriptional regulation, and ensuing downstream gene expression is increasingly evident. Adequate and controlled Cn/NFAT signaling is necessary, as dysregulation results in several vascular pathologies. Both the

versatility in DNA binding and the presence of multiple NFAT family members suggest NFAT proteins have a more extensive role than originally anticipated. A few notable features of NFAT are brought to attention below.

NFAT transcription factors bind to DNA in a rather versatile manner. The promiscuous nature of NFAT-DNA binding suggests the presence of other binding partners. The most common and well-documented binding partner of NFAT is AP-1, a transcription factor comprised of Fos- and Jun- family proteins (Rao, Luo & Hogan, 1997). Several groups have demonstrated the need for cooperative NFAT-AP1 binding for optimal target gene transcription (Hogan *et al.* 2003). In addition to AP-1, NFAT has been shown to interact with other transcription factors involved in cell growth and differentiation, including GATA4, MEF2, and FOXP3 (Crabtree & Olson, 2002; Wu *et al.* 2006). Several groups have also noted the presence of multiple NFAT binding sites in validated NFAT-target gene promoters, such as interleukin-2 (Randak *et al.* 1990), interleukin-4 (Chuvpilo *et al.* 1993), and DSCR1 (Harris, Ermak & Davies, 2005). These multiple NFAT binding domains most likely confer a synergistic effect between NFAT-containing complexes and thereby promote efficient gene transcription (Rao *et al.* 1997). Thus, NFAT proteins may serve as 'coincidence detectors' (Crabtree & Olson, 2002) that can integrate information from multiple signaling pathways to coordinate gene expression.

The ubiquitous nature of NFAT amongst various cell types and processes demonstrates the broad-range effects of NFAT activity. While functional redundancy has been noted between the different NFAT isoforms, differences in tissue distribution (Rao *et al.* 1997), evidence of multiple splice variants, and distinguishing phenotypes of NFAT-deficient mice suggest NFAT proteins may in fact possess unique isoform-specific functions. For example, while NFATc1 and NFATc2 share 72% sequence agreement and demonstrate functional similarities, genetic deletion for either NFATc isoform results in divergent mouse phenotypes. Whereas NFATc2^{-/-} mice are viable and demonstrate an enhanced immune response (Duque, Fresno & Iniguez, 2005a), NFATc1^{-/-} mice display abnormal cardiac valve development and consequent embryonic lethality (de la Pompa *et al.* 1998). Variations between NFAT proteins is further seen where targeted disruption of NFATc3, but not NFATc4, significantly reduces Cn transgene-induced cardiac hypertrophy (Wilkins *et al.* 2002). The disparate outcomes from NFATc genetic deletions as well as the ability for NFATc isoforms to compensate one another limits the use of NFATc-specific knock-out mice.

Recent data have also challenged the longstanding premise viewing NFAT as a transcriptional activator. Several groups have in fact demonstrated the dual role of NFAT as both a gene activator and silencer (Robbs *et al.* 2008). NFATc1 and NFATc2 have been shown to repress cyclin-dependent kinase 4 (Horsley *et al.* 2008) and cyclinA2 (Carvalho *et al.* 2007) expression, respectively. NFATc2 has also been demonstrated as a negative regulator of cyclin B1 and cyclin E in lymphocytes (Caetano *et al.* 2002). The nonoverlapping functions and apparent gene silencing effects of individual NFATc proteins further complicates our understanding of Cn/NFAT signaling. NFAT is clearly involved in maintaining a balance between cell quiescence and cell proliferation – the two fundamental biological mechanisms underlying tumorigenesis and atherosclerosis.

7. Conclusions

The Cn/NFAT signaling cascade is undoubtedly a major determinant of cell growth and differentiation. Regulation of Cn/NFAT signaling is a critical determinant of neoplastic and

cardiovascular risk. With DSCR1 now known as an endogenous regulator of Cn/NFAT signaling, several groups have successfully manipulated downstream gene expression and cell function by altering DSCR1 levels. The observation that DS patients exhibit significantly less tumor growth and arterial plaque formation cannot be mere coincidence, but rather evidence supporting the notion that these large-scale, vascular-related pathologies share an underlying mechanism. This is further reinforced by the fact that DSCR1 and other candidate antiangiogenic genes found on hChr21 are of growing attention. A more comprehensive analysis of hChr21-located genes may therefore provide direction as we continue to elucidate the mechanisms underlying tumorigenesis and atherosclerotic plaque formation.

8. References

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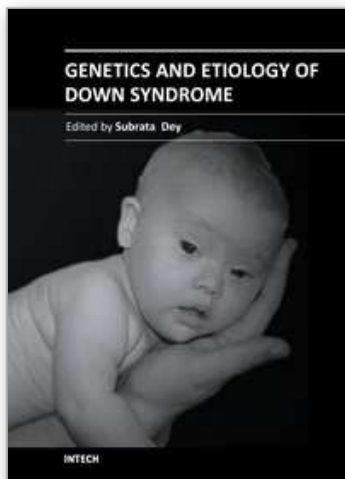
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This book provides a concise yet comprehensive source of current information on Down syndrome. Research workers, scientists, medical graduates and paediatricians will find it an excellent source for reference and review. This book has been divided into four sections, beginning with the Genetics and Etiology and ending with Prenatal Diagnosis and Screening. Inside, you will find state-of-the-art information on: 1. Genetics and Etiology 2. Down syndrome Model 3. Neurologic, Urologic, Dental & Allergic disorders 4. Prenatal Diagnosis and Screening Whilst aimed primarily at research workers on Down syndrome, we hope that the appeal of this book will extend beyond the narrow confines of academic interest and be of interest to a wider audience, especially parents and relatives of Down syndrome patients.

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