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Exploring the role of TCF1 expression in Smooth Muscle Cells and its relationship with Coronary Heart Disease

1. Importance and challenges of atherosclerotic coronary heart disease

Atherosclerotic coronary vascular heart disease (CHD), which clinically is the cause of acute myocardial infarctions, is the primary cause of morbidity and mortality in the Western civilized world[1]. While great progress has been made with regard to the identification and treatment of this disease, the majority of disease remains untreated and uncontrolled. New research approaches are required to develop more fundamental understanding of the atherosclerosis disease process, so that more specific treatments can be developed.

Focus on cardiovascular disease began around the time of the death of President Franklin Delano Roosevelt, who suffered from hypertension, stroke and CHD. Funds were appropriated by Congress to establish classical longitudinal epidemiological studies to identify risk factors that were associated with the development of CHD. The Framingham Heart Study was one of these, and enrolled subjects in the town of Framingham, Massachusetts for monitoring. Numerous demographic, behavioral and easily measured physiological parameters were catalogued, and subjects followed and carefully monitored to search for "risk" factors and cardiovascular and related medical conditions such as myocardial infarction. This work provided tremendous insight insight into the development of CHD, allowing the identification of highly associated risk factors. Risk factors such as hypertension, hypercholesterolemia, and smoking were identified and quantified, and a Framingham Risk Score developed that provided for assessment of the likelihood that individuals with a specific complement of demographic features and risk factor profile would develop symptomatic CHD[2]. While primarily useful in the context of studies comparing overall risk between groups at a population level, the Framingham Risk Score remains in use today as the best means for identifying and providing some measure of guidance regarding the assessment and treatment of

individual patients.

With this information regarding risk factors, pharmaceutical firms leveraged scientific insights coming from molecular research to develop drugs targeting physiological pathways to normalize risk factor variables such as serum cholesterol and blood pressure. These medications, such as HMG-CoA reductase inhibitors and angiotensin converting enzyme inhibitors have been able to significantly reduce the incidence of myocardial infarction and CHD-related endpoints in numerous clinical trials, providing great progress in the treatment of this disease[3, 4]. However, aggressive control of cardiovascular risk factors with behavioral intervention and optimum medical treatment cannot lower event rates by more than 30%. Thus, the majority of cardiovascular events cannot be prevented by currently available treatments. These limitations of risk factor treatment are not surprising when one considers that while risk factors are important for promoting CHD, they are not the actual atherosclerotic disease process in the blood vessel wall. Numerous cellular and molecular pathways have been characterized in the diseased vascular wall, and linked to various aspects of disease with model systems and in vitro methods. Over the last decade, there has been a consensus that inflammatory processes underlie atherosclerosis[5]. However, there is in fact little compelling data in humans regarding which specific pathways drive the disease, and pharmaceutical firms have been reluctant to pursue expensive and risky studies to develop drugs that treat the primary atherosclerotic disease and today there no such drugs available.

2. Use of human genetic studies to identify genes and pathways that mediate human CHD

Thus, the greatest need for further progress for treating CHD relies on the identification of pathways in the vessel wall that mediate the initiation and/or progression of disease. The greatest promise for identifying such pathways is through the identification of human genetic variation that alters the risk for CHD in human populations. Such variation represents an experiment of nature that validates unequivocally that altering the expression and/or function of the related gene alters the risk of disease. Drugs directed against such pathways are expected to provide

therapeutic benefit. Also, heritability studies suggest that approximately half of the risk of CHD is genetic in nature, as witnessed by the common observation that this disease clusters in families [6, 7]. This heritable risk is independent of the classical epidemiological risk factors, and most likely represents variation in pathways that mediate the atherosclerotic disease process [6].

Although methodology and technology for identifying CHD-associated variation in the human genome have been long unavailable, recent developments including the human HAPMAP project and high density genotyping chips have provided for large-scale mapping studies that have identified variation that contributes to the genetic risk for CHD in the human populations[8-11]. The majority of these variants are not easily linked to identifiable genes, or appear to be associated with genes that have functions not easily linked to the vascular wall. However, a small number of the genes identified through Genome Wide Association studies (GWAs) efforts provide immediate testable hypotheses that promise to teach us much regarding the pathophysiological basis of atherosclerosis.

One such gene is TCF21. TCF21 is expressed in progenitor cells of the proepicardial organ (PEO) in the early embryo, and these cells are known to be the source of coronary smooth muscle cells (SMC) in this unique circulatory bed[12]. Recent data from the Quertermous lab with adult mice carrying a $TCF21^{lacZ}$ reporter allele shows that TCF21 expression persists in coronary vascular SMC. It has been hypothesized that TCF21 in the PEO supports proliferation and inhibits differentiation of SMC progenitor cells as they migrate over the surface of the heart until they come under local inductive influences that downregulate TCF21 expression[13]. These hypotheses are based on available information regarding the role of TCF21 in cell fate decisions. TCF21 expression is downregulated with differentiation of PEO cells in vivo and in SMC differentiated in culture. Transcriptional regulation by TCF21 has been shown to promote cell division of skeletal muscle cells by inhibition of transcription of the cyclin dependent kinase inhibitor p21CIP, and inhibit differentiation by inhibiting transcription of the differentiation marker muscle creatine kinase[14].

3. Proposal to study the role of TCF21 in vascular smooth muscle cell fate decision

SMC play critical but complex roles in vascular disease, undergoing phenotypic switching that is related to multiple cell fate decisions[15, 16]. In the face of vascular injury, SMC de-differentiate, migrate and then re-differentiate. While SMC promote disease through producing neointimal lesions and becoming foam cells, they also serve an important supportive role through production of stabilizing matrix components, and cardiovascular events due to plaque rupture may be in lesions where SMC response to injury is inadequate, "fragile plaque." While it is generally accepted that mutations that compromise the ability of SMC to participate in vascular repair are likely detrimental to the organism[16], the exact mechanism by which TCF21 might participate in the physiological SMC response to injury, and how this is perturbed by differences in TCF21 expression remains to be elucidated. It is considered most likely that activation of TCF21 expression in the adult in the face of vascular injury promotes a maladaptive SMC contribution to repair mechanisms. A critical first step to understanding how TCF21 modulates the SMC response to injury, and thus likely the in vivo disease process, is to pursue in vitro studies in order to gain understanding of what role this gene plays in the critical cell fate decisions that are important in the SMC response to injury. Thus, in our proposed studies we will manipulate expression of TCF21 to better understand how this gene differentially regulates disease related processes in SMC such as proliferation, differentiation, migration, and apoptosis. The Quertermous laboratory has extensive experience with in vitro cellular and molecular studies in vascular cells such as those proposed here[17-20].

Experimental Methodology- For these studies, we will use an in vitro tissue culture model that employs human coronary artery SMC (HCASMC). These cells, which are commercially available, can be easily expanded and grown in culture, can be studied in an "undifferentiated" or disease state when grown in the presence of serum, or in a quiescent serum starvation state which models the physiological "differentiated" phenotype. Preliminary studies in the Quertermous lab has shown that TCF21 expression is ~5-fold higher in HCASMC grown under the proliferative phenotype. These cells can be easily transfected by electroporation with the Amaxa system.

We will use human expression constructs that encode the native transcript for

human TCF21 to increase mRNA levels of the gene in transfected HCASMC. shRNA constructs that target human TCF21 have been developed by the Broad shRNA Initiative (Sigma, Open Biosystems), and will be transfected into HCASMC to decrease the level of TCF21 mRNA. Empty expression constructs will serve as negative controls in these experiments. In each of the genetic manipulations, we will monitor by quantitative polymerse chain reaction (qRT-PCR) the mRNA levels of TCF21 and specific identified downstream targets of TCF21.

Transfected cells will be grown in either serum (proliferating disease model) or serum-free differentiation media (quiescent non-disease model), and proliferation evaluated by cell counting and MTS assay. SMC differentiation will be evaluated with qRT-PCR assays to quantify expression of SMC differentiation markers, including SMC α-actin, myosin and transgelin. Particular attention will be given to survival pathways, apoptosis will be evaluated using the Caspase-Glo 3/7 Assay (Promega) and Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay for microscopy (Invitrogen), employing serum starved HCASMC exposed to 1 mM staurosporine. Finally, migration will be evaluated with genetically modified cells in the Boyden chamber assay, employing migration in response to various dilutions of serum, with well described methods[18].

It is anticipated that alterations in TCF21 expression level will significantly impact cell fate decisions such as proliferation, differentiation and apoptosis. TCF21 expression will likely be found to promote SMC proliferation and inhibit differentiation. Also, loss of TCF21 is expected to increase apoptosis, in keeping with evidence of increased apoptosis in TCF21 knockout mice[21, 22]. These studies will teach us much regarding the biology of SMC and the contribution of this cell type to the different phases of vascular disease, and insights gained will extend beyond the role of this single factor in the mechanisms of disease initiation and/or progression.

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