Investigating smooth muscle phenotypic transition in human vascular disease to identify disease-promoting mechanisms, prognostic biomarkers and therapeutic targets

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Background: The progression of cardiovascular diseases, such as atherosclerosis, systemic and pulmonary hypertension, diabetic vasculopathy and aortic aneurysm, is critically determined by the response of smooth muscle cells (SMCs) within the arterial medial layer. In their fully differentiated, contractile state, SMCs confer stability and regulate vascular tone. In disease, however, growth factors, notably platelet-derived PDGF-BB secreted by damaged endothelium and immune cells, induce a 'contractile-synthetic' switch in SMCs. This phenotypic transition is an intrinsic property of SMCs, enabling them to respond to physiological and pathophysiological stimuli and actively participate in tissue remodelling and repair processes. However, chronic SMC dedifferentiation impairs contractile function, leads to vascular thickening and stiffness and exacerbates inflammation, to promote atherosclerotic plaque formation and susceptibility to aneurysmal dilatation[1-5]. The switch is characterised by deterioration of the contractile apparatus, with loss of markers such as α-smooth muscle actin (*ACTA2*), smooth muscle-myosin heavy chain (*MYH11*), transgelin (*TAGLN*) and calponin (*CNN1*), and alteration of cellular behaviour. Markers associated with synthetic phenotypes, including metalloproteinase-9 (*MMP9*) and osteopontin (*OPN*), reflect the altered matrix secretion and enhanced migratory capacity.

Modulated SMCs have been proposed to give rise to multiple distinct phenotypes in the fibrous cap and necrotic core, to profoundly influence plaque morphology and stability[6]. Fibroblast-like, mesenchymal, osteogenic, macrophage-like, and adipocyte-like populations have been described (Figure 1)[4]. While some may stabilise the fibrous cap, most exacerbate inflammation and promote apoptosis, extracellular matrix remodelling, and calcification, ultimately increasing plaque size and vulnerability[7]. While inhibiting SMC phenotypic transformation has proven potential to attenuate progression of vascular disease in animal models[1, 2, 8], our limited knowledge of the underlying mechanisms has resulted in a paucity of therapeutic targets. Moreover, the low success rate in translation from animal studies to the clinic highlights the need to determine whether similar mechanisms serve to protect the human vasculature, and how they may be targeted to alleviate disease. Genome-wide

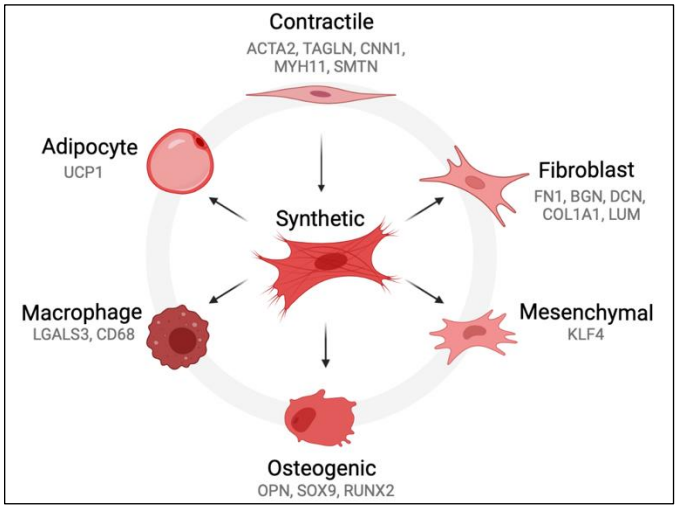

***Figure 1. Schematic detailing the variety of synthetic SMC phenotypes and their associated markers**. Created with BioRender.com, based on[4, 6].*

association studies (GWAS) have yielded insights into genetic predisposition e.g. mutations in the atheroprotective endocytic receptor, *LRP1* or transcription factor, *TCF21*. However, the challenge in the 'post-genomic era' remains to elucidate the molecular mechanisms through which GWAS 'hits' influence pathogenesis and to identify targets for disease prevention. Treatment options are further confounded by the lack of prognostic biomarkers based on SMC phenotype, rather than inflammation, to predict clinical trajectory.

Pilot data: To address these limitations, we established a human *in vitro* model of disease-induced coronary arterial SMC (hcSMC) phenotypic transition. We benchmarked against scRNA-seq data of diseased human coronary arterial plaques[2] to validate the accuracy and predictive power of the model, and importantly we have scaled to a 384-well plate format for medium/high throughput semi-automated screening. Transcriptomic analysis of the hcSMC transition has already revealed putative novel drivers of SMC dedifferentiation, as well as downstream mechanisms underlying the switch (Figure 2).

Hypothesis: ***Our robust hSMC model will enable the investigation of disease-relevant mechanisms and markers, and screening to identify therapeutic targets for coronary artery disease (CAD)***.

Aims of the Project: *1) To delineate key mechanisms driving modulation of SMC phenotype and disease progression. 2) To identify prognostic biomarkers of SMC dedifferentiation. 3) To identify targets for treatment of CAD.*

Description of the work to be undertaken:

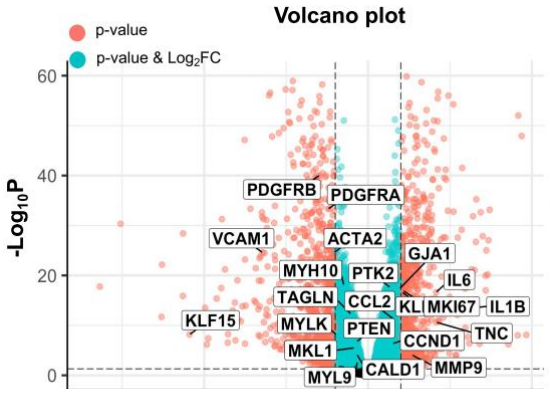

Figure 2: RNA-seq analysis of the in vitro hcSMC disease model. *Differentially expressed genes 24 hours after simulated disease. Previously implicated genes are highlighted. ~1000 novel genes were additionally identified, including ~200 GWAS loci.*

Aim 1. Medium-throughput targeted screens (*Digital RNA with pertUrbation of Genes*; DRUG-seq)[9] will be performed, using siRNA against selected regulators identified from the RNA-seq of hcSMC phenotypic modulation. Priority will be given to prominent CAD GWAS-implicated genes that were differentially expressed, as well as prominent transcription factors induced early upon simulated disease. Significantly altered marker expression and morphological alterations identified by automated imaging will be the primary readouts, alongside transcriptome profiling, to capture the transcriptional diversity in an unbiased manner. Such comprehensive transcriptomic readouts will allow us to distinguish downstream effectors that are common across genetic perturbations, in order to define core pathways of disease progression. These will be evaluated in relation to CAD-associated loci using GWAS summary information (derived from a case-control sample size of over 1 million). We will cross-reference against the wealth of published scRNA-seq data sets from a range of murine and rat disease models, as well as from healthy and diseased human arteries. Comparison across data sets will reveal common, as well as organotypic, disease-specific, SMC molecular traits associated with phenotypic modulation. Where novel pathways are identified, we will seek to validate altered gene expression in existing murine disease samples, and in clinical samples e.g. from the OxAMI cohort. Mechanisms will be further elaborated *in vitro* using assays appropriate to the pathways and, for selected key mechanisms, validation will extend to human coronary micro-artery samples[10].

Aim 2. Proteomic analysis of the SMC secretome will be performed, to detect novel biomarkers associated with the loss of contractile function. Enhanced secretion in disease will be correlated with altered transcript levels in human CAD scRNA-seq. Prognostic utility, ultimately for patient

stratification and monitoring efficacy of treatment, will be assessed in the first instance by proteomic analysis of serum from the murine CAD study (Aim 3) and validated in clinical cohorts.

Aim 3. Using transcriptomic data from the DRUG-seq screens in Aim 1, we will use a computational pharmacogenomics approach to identify candidate compounds that target the relevant mechanisms. The Connectivity Map[11, 12] associates human diseases with underlying genes and potential therapeutic drug candidates, and has been successfully applied to DRUG-seq outputs[9]. A list of key genes relevant to phenotypic switching will be compiled and used to derive a list of drugs with putative efficacy for preservation of SMC phenotype. A targeted small molecule screen will assess efficacy in attenuating SMC dedifferentiation in the *in vitro* disease model, with compounds prioritised based on pharmacological properties, safety, and disease relevance. While target validation in human cell-based systems will ensure translational relevance, our expertise will allow for *in vivo* testing of leading candidate therapies in murine models of atherosclerosis. A pilot study will initially be performed and, if safety and efficacy demonstrated, we will work towards larger scale preclinical testing and clinical translation. While the central focus of this project is on CAD, we will additionally explore, via analysis of existing murine disease samples and published human data sets, whether the mechanisms and targets may also be appropriate for SMC preservation in other relevant diseases e.g. AAA, pulmonary hypertension.

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