Smooth muscle phenotypic modulation in atherosclerosis

Atherosclerosis is a complex inflammatory process in which plaque composed of lipids, matrix proteins, smooth muscle and inflammatory cells accumulate in the intima of mid to large sized arteries. Atherosclerosis is the main disease process underlying highly morbid and lethal diseases like myocardial infarction (MI) and stroke. Plaques are a slow-growing phenomenon that manifest early in life, first as a thickening of the intima in which the plaque is rich in smooth muscle cells (SMCs), extracellular matrix (ECM) and focal lipid accumulation. Later, plaques grow to a more pathologic fibroatheroma which includes macrophage infiltration, foam cells, a large core of necrosed cellular and matrix debris and an overlying fibrous cap. Late stage plaques can destabilize, wherein the fibrous cap thins and ruptures, exposing the thrombogenic contents of the necrotic core to circulation and provoking late stage sequelae, like MI or stroke.

Plaques destabilize due to a loss of matrix synthesizing SMCs and fibroblasts and accelerated degradation the fibrous cap owing to macrophage secreted extracellular proteases (Silvestre-Roig et al., 2014). In the healthy vessel, SMCs are located in the medial layer and are responsible for arterial contraction and ECM synthesis. In early stages of atherosclerosis, vascular SMCs promote disease by proliferating aberrantly which promotes plaque formation (Silvestre-Roig et al., 2014). In advanced plaque, a subset of SMCs de-differentiate and lose their contractile protein expression and myofilament density, a process called phenotypic modulation. Lineage tracing studies have demonstrated that SMCs can modulate their phenotype to resemble myofibroblasts, macrophages, foam cells and osteochondrogenic cells (Hao et al. 2006; Shankman et al. 2015; Allahverdian et al. 2014; Kapustin et al. 2015). Until recently, phenotyping de-differentiated SMCs relied on searching for cell specific markers expressed by lineage traced SMC progeny using immunostaining or qPCR. These approaches provided some idea of the fate of phenotypically modulated SMCs but required a biased search strategy, as researchers would probe with predetermined cell-specific markers. This approach failed to characterize up to half of the cell population (Shankman et al. 2015; Allahverdian et al. 2014). With phenotypically modulated SMCs achieving such distinct cell fates, such as macrophage- and myofibroblast-like cells, their role as protective or pathogenic to atherosclerosis is not clear.

Recently, Wirka et al. (2019) published a study that utilized single cell-RNA sequencing to reveal that the phenotypically modulated SMC population in mice may be more transcriptionally homogenous than once thought. They demonstrated that phenotypically modulated SMCs polarize almost exclusively to a fibroblast-like cell, termed a ‘fibromyocyte’, rather than multiple unique cell types. In fact, they found that these fibromycotes do express many of the cell-specific markers identified in previous studies but do so only transiently. The fibromyocyte transcriptome includes *Fn1, Col1a2, Tnfrs11b, Lum and Timp1*, among other genes, in mouse atherosclerosis tissue. This study also showed that SMC-specific deficiency of the *Tcf21* gene resulted in a smaller fibromyocyte population and thinner fibrous cap. TCF21 has been shown in both quantitative trait locus studies (Liu et al. 2018; Miller et al. 2013) and mechanistic studies (Nurnberg et al. 2015; Sazanova et al. 2015) as the causal gene in the protective CAD-associated locus at 6q23.2. Finally, coronary artery biopsies from heart transplant patients demonstrated TNFRSF11B, a gene associated with mouse fibromyocytes, expression in the fibrous caps of human plaques (Wirka et al. 2019)

This new evidence that murine vascular SMCs phenotypically switch into fibromyocytes, a single cell population with a transcriptional profile distinct from macrophages and one more similar to fibroblasts is extremely compelling. I posit that this cell population is atheroprotective and functions to stabilize late stage lesions by strengthening the fibrous cap. To explore this further I propose the following three questions:

Does an expanded fibromyocyte population stabilize advanced atherosclerotic lesions in mice?

Wirka et al. demonstrated that mice lacking *Tcf21* in SMCs underwent less SMC phenotypic modulation and had less SMC and fibromyocyte migration to the fibrous cap. In vitro studies showed that *Tcf21* overexpression in human coronary artery SMCs resulted in increased fibromyocyte transcriptome expression. Most investigations into SMC phenotypic modulation have performed in atherogenesis models, rather than plaque rupture models. What remains to be

seen is if (1) overexpressing *Tcf21* in SMCs can actually expand the fibromyocyte population and (2) whether increasing fibromyocyte levels either before or during disease onset can ameliorate late stage atherosclerosis using plaque rupture models. Here I'll discuss several approaches to conduct a study like this.

There is no reported transgenic mouse line that overexpresses *Tcf21* in SMCs specifically, though in theory, various strategies exist to create this. The most direct strategy would be to supply *Tcf21* linked to a *Myh11* fragment promoter (a very specific SMC marker) to the vascular wall using adeno- or adeno-associated virus. In theory, if this virus is efficiently delivered to the medial SMC layer of the vessel, SMCs will express higher levels of *Tcf21*. A major limitation is efficient viral delivery to the medial layer of the vessel wall. Doing this often involves an intraluminal balloon catheter that disrupts the endothelium to access SMCs (Ding et al. 2004). Doing this may inadvertently elicit other cellular processes, like intimal hyperplasia.

Another approach to manipulating gene expression in a cell-type specific manner that does not require intravascular intervention is the tamoxifen inducible bacterial Cre-LoxP recombinase system. In this system, Cre protein expression can be induced by administering tamoxifen and Cre then catalyzes recombination between two lox sites, excising the gene flanked by the lox genes (Feil et al. 1997). Again, this could occur in SMCs specifically by having the *Myh11* promoter drive Cre expression (Regan et al. 2000). However, in order to achieve *Tcf21* overexpression, the Cre-LoxP system would need to excise a *Tcf21* transcriptional repressor. There are a few reports of *Tcf21* transcriptional inhibitors, including miRNA-3648 (Sun et al. 2019) and miRNA-224 (Miller et al. 2014).

A third approach may be to directly supply oligonucleotides or antisense oligonucleotides that regulate *Tcf21* expression to the medial layer (again using a viral vector approach). There are a few reports of long non-coding RNAs which upregulate *Tcf21*, like TARID (Arab et al. 2014) and LINC00163 (Guo et al. 2018). Directly administering these lncRNAs could increase *Tcf21* expression in vascular SMCs in vivo. As mentioned, miRNAs -3648 and -224 are associated with suppression of *Tcf21* expression. Administering antisense oligonucleotides to inhibit these microRNAs may lead to *Tcf21* expression in vascular SMCs.

The goal of these three approaches would be to elicit *Tcf21* expression in SMCs specifically in vivo, in order to expand the baseline fibromyocyte population either before or after induction of atherogenesis. This would help us see if fibromyocytes can protect plaque from destabilizing. Of the three methods listed, virally supplying *Myh11* linked *Tcf21* to the medial layer, while not perfect, is the most direct approach and does not require complicated breeding schemes. To measure whether doing this actually expands the fibromyocyte population, the arterial tissue could be examined histologically for transcriptional markers of fibromyocytes.

For the next step in this study, it is critical to select an appropriate atherosclerosis model. Preclinical murine models for atherosclerosis provide a fast growing, reproducible, cost-effective disease model in which whole body and cell specific genetic manipulation is possible. However, most mouse model lesions fail to capture arguably the most clinically relevant feature of advanced atherosclerotic lesions - plaque rupture. The *ApoE*-/- Fbn1C1039G+/- is among the more efficacious models for plaque rupture. It is achieved by cross-breeding ApoE deficient mice with mice heterozygous for a fibrillin-1 mutation in combination with a high fat diet. This model requires only 20 weeks to develop and 70% of mice display plaque rupture, resulting in MI, stroke or sudden death. Histopathologically, these lesions display a large necrotic core, robust macrophage infiltration, neovascularization and intra plaque hemorrhage, all features of unstable human plaques (Van Herck et al. 2009).

In summary, to explore whether fibromyocyte expansion can stabilize advanced plaque, I propose using a viral vector to overexpress *Tcf21* in a SMC specific manner in *ApoE*-/- Fbn1C1039G+/- mice and induce atherosclerosis with a high-fat diet. Measurable outcomes include thromboembolic complication (MI, stroke, sudden death) rates and and the above listed histologic features of plaque instability in the aortic root, arch and carotid arteries, where this model is

known to produce plaque. Histologic examination of fibromyocytes could be achieved using in situ RNA hybridization to probe for genes unique to the fibromyocyte transcriptome, such as *Tnfrs11b*, *Lum* and *Timp1*.

Do fibromyocytes exhibit the matrix contracting function of fibroblasts in vitro?

The fibrous cap of fibroatheroma is primarily composed of collagen and SMCs and functions to envelope the highly thrombogenic necrotic core, shielding the core's contents from entering into circulation. High collagen content and thickness contributes to a stable plaque. As discussed earlier, phenotypically modulated SMCs adopt a fibroblast-like transcriptome expressing ECM genes, like *Col1a2* and *Fn1* and genes associated with tissue remodeling, like *Timp1*, however, the function of this newly described cell type is not yet characterized.

Human coronary artery SMCs can be phenotypically modulated into fibromyocytes by overexpressing *Tcf21* using lentivirus. I propose using biomechanical functional assays to test whether fibromyocytes have the capacity to contract surrounding matrix in vitro, a process similar to wound healing in vivo. One option for this is to use a free-floating fibromyocyte-populated collagen lattice, a well established assay designed for fibroblasts that relies on optical measurements to detect collagen lattice shrinkage, a sign of contraction (Bell, Ivarsson, and Merrill 1979). A study published this year detailed a newly designed dynamic microscale gel contraction system which requires significantly fewer cells and collagen, has straightforward measurements of gel contraction that does not require manipulation of the gel (a large source of error in the conventional, free-floating assay), and is compatible with co-culture experiments (Zhang et al. 2019). Seeding fibromyocytes onto a microscale gel contraction system like this could help elucidate whether these cells contribute to matrix remodeling via contraction. This assay, however, would not give insight into migration and matrix degrading properties of the cell, which are other critical functions of cells that remodel tissue.

Do fibromyocyte levels in human coronary artery samples differ in patients with stable and unstable plaque?

One study has already shown that human coronary plaque has evidence of a greater fibromyocyte levels (Wirka et al. 2019). Next, I would like to see if fibromyocyte levels differ in stable versus unstable human plaque. To do this one could obtain human coronary artery tissue biopsies from a tissue bank (e.g. the Sydney Heart Bank) from patients who either suffer an occlusive MI (unstable plaque) and from patients with "stable plaque", then comparing fibromyocyte levels in these groups. How to define tissue donors with "stable plaque" is a challenge. Clinically, stable coronary artery disease refers to patients who have not had a recent acute event (MI, unstable angina). Clinical stability, however, does not necessarily reflect plaque stability. A given patient with a stable clinical picture can still be at high risk for future cardiac events. Nevertheless, obtaining coronary samples from tissue donors with known CAD but with no history of unstable angina, MI or stroke would be a nonspecific way to screen tissue as potentially having stable plaque. From there, one could examine the tissue histologically for features of stable plaque lesions(thick fibrous cap, higher SMC population, fewer macrophages). Assessing TNFRSF11B levels in coronary tissue from patients with known ruptured plaque and patients with asymptomatic atherosclerosis and histologically stable appearing lesions could help further discern if fibromyocytes are associated with plaque stability.

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The presence of phenotypically modified SMCs in atherosclerosis was first detected decades ago. While originally seen as a protective cell type, years of lineage tracing studies and immunostaining based phenotyping approaches cast doubt on this- identifying a subset of these cells that harbor disease exacerbating macrophage-like properties. Single cell RNA sequencing elucidated that modulated SMCs are potentially be a homogenous fibroblast-like cell type in mice, raising further question on the pathologic or protective role of this cell process. Embryonically expressed Tcf21 in part drives SMC phenotypic modulation. To assess the therapeutic benefit of this phenomenon, we must understand if increasing its expression actually protects against CAD, as proposed in the first question. In this essay I also described investigating this in human tissue and proposed an in vitro study to better understand the cellular functions of these modulated SMCs.

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