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Reconciling smooth muscle cell oligoclonality and proliferative capacity in experimental atherosclerosis

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Despite decades of research, the relative contribution of vascular smooth muscle cells (SMC) to the pathogenesis of atherosclerosis has remained unclear and controversial. Most of the controversy came from the lack of rigorous lineage tracing studies to unequivocally identify the cellular origins of lesion cells. Indeed, as extensively described in recent reviews[1, 2], the plasticity of multiple lesion cell types (SMC, myeloid cells, and endothelial cells), their ability to downregulate their "lineage-specific" markers and to express markers of alternative cell types during atherosclerotic plaque progression makes their identification using traditional immunostaining for such markers highly inconclusive and greatly confounds attempts to understand the functional roles of these cells within lesions. With respect to SMC, tremendous progress has been made since the development of rigorous and unambiguous murine lineage tracing systems allowing conditional, inducible, definitive and efficient labelling of medial Myh11^{+} SMC and tracking of their fate in atherosclerosis as well as simultaneous SMC-specific conditional knockout of genes postulated to control SMC phenotype and overall lesion pathogenesis. Using these capabilities our lab[3, 4] recently demonstrated that: 1) >80% of SMC-derived cells within advanced lesions of atheroprone ApoE$^{-/-}$ mice fed a western diet for 18 weeks lacked detectable expression of SMC markers such as Acta2 or Myh11 typically used to identify them; 2) Myh11^{+} medial SMC can undergo multiple phenotypic transitions characterized by activation of markers of macrophages, mesenchymal stem cells, and myofibroblasts; and 3) contrary to the long standing dogma that SMC play a beneficial role in lesion pathogenesis by contributing to the formation of a protective fibrous cap, results from our recent studies in which we performed SMC-specific conditional KO of the pluripotency factors Klf4 and Oct4 demonstrated that SMC can play either an atheroprotective or atheropromoting role depending on the nature of their phenotypic transitions[3, 4]. For example, Klf4-dependent transitions, including formation of SMC-derived macrophage-marker^{+} foam cells exacerbated lesion

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pathogenesis[3] whereas Oct4-dependent transitions were atheroprotective including being critical for migration and investment of SMC into the fibrous cap[4]. However, our studies failed to clearly resolve a number of critical questions. *First,* are SMC-derived cells that populate lesions derived from many or only a few differentiated medial SMC, a hypothesis originally proposed by Earl Benditt in 1973 in his "monoclonal theory of atherosclerosis"[5]? *Second,* can a single differentiated medial SMC give rise to multiple SMC phenotypes or are individual SMC limited in their plasticity?

In the present issue of *Circulation Research*, Chappell et al.[6] elegantly address each of these key questions by using a SMC-specific multi-color confetti mouse to demonstrate conclusively that the SMC within both atherosclerotic lesions and neointima following vascular injury originate from a small subset of medial Myh11$^+$ SMC. Moreover, they provide evidence that SMC derived from a single clone can give rise to both fibrous cap Acta2$^+$ SMC and necrotic core Mac3$^+$ SMC. Before further considering the very important implications of these findings, it is important for us to clarify several often misunderstood nuances, merits, and limitations of the different lineage tracing systems and their applications.

Clonal analysis systems as employed by Chappell et al.[6] and previously by Feil et al.[7] are very powerful for ascertaining the fate of individual cells, in these cases Myh11 or SM22 expressing medial SMC respectively. However, clonal analysis systems are not compatible for assessing the DIRECT overall contribution of a given cell type or gene/pathway to lesion pathogenesis by combining inducible SMC clone tracing with SMC-specific knockout as used in our previous studies[3, 4]. The reasons are several-fold. *First*, clonal analysis systems rely on having incomplete stochastic recombination rates (e.g. 11% in Feil et al.[7], 1% and 78% for low and high density labeling respectively in Chappell et al. paper[6]) in order to achieve unambiguous spatial resolution of clones. However, this of course is incompatible with attempting to rigorously identify all or nearly all SMC-derived lesion cells as was the goal of our studies[3, 4]. *Second*, although clonal tracing systems can be used effectively in combination with KO strategies to define how a particular gene regulates SMC clonal expansion, selection and/or phenotypic transitions, they have limited sensitivity and/or provide confounding results in ascertaining how loss of that gene in SMC impacts overall lesion pathogenesis. Indeed, the presence of a large subset of non-Cre recombined wild type and unlabeled SMC will likely: 1) significantly reduce the possibility of seeing an overall effect on lesion pathogenesis due to compensation of non-recombined wild-type SMC; and/or 2) artificially bias results if loss of the candidate gene of interest confers a survival advantage (or disadvantage) to that subset of cells. Indeed, as the results of Chappell et al.[6] nicely show, even a small number of residual wild type cells might undergo selective clonal expansion or selection and end up being the dominant cell type within lesions. This knowledge could be extremely valuable, but unfortunately is unlikely to provide insights regarding mechanisms that normally regulate overall lesion pathogenesis. In addition, it is unlikely to identify potential novel therapeutic approaches for treating the disease since it may be difficult or impossible to develop ways to selectively target only certain clones. In conclusion, we want to emphasize that, rather than being mutually exclusive, the clonal tracing and the high-efficiency lineage tracing systems should be used complementarily to answer different questions.

Whereas the data presented by Chappell et al.[6] are compelling with respect to the oligoclonality of lesion SMC, these studies cannot discriminate between clonal expansion, clonal selection or a combination of the two. Indeed, the authors infer that only a small subset of SMC retain proliferative capacity and undergo clonal expansion based on making several untested assumptions including: 1) that the number of clones observed at the end point is equal to the number of clones that initially proliferate and/or migrate into lesions; and 2) that only the subset of medial SMC that give rise to clones within atherosclerotic lesions underwent cell proliferation during the entire process of atherosclerosis development. An analogy of this over-interpretation would be to erroneously conclude that the number of adult salmon returning to fresh water are derived from an equivalent small number of young salmon smolt entering the ocean several years before. Although millions of young salmon smolt leave freshwater and enter the ocean, only a tiny fraction return as adults to spawn and give rise to the next generation of salmon due to various “selection” processes. A similar process of clonal selection, rather than limited proliferation, may account for the oligoclonal nature of neointimal SMC. Indeed, to directly assess the number or proportion of medial SMC that initially proliferate and give rise to lesion SMC would require rigorous estimation of the medial SMC growth fraction (GF) as employed in classic studies by Alexander Clowes[8], and Wilbur Thomas[9]. In brief these studies involved determining the fraction of medial cells (presumably mostly SMC) present at time zero that undergo DNA synthesis at any time point following either rat carotid balloon injury or porcine atherosclerosis. Of major relevance to the Chappell et al. studies, they reported a growth fraction for medial cells of nearly 100% and 40% in porcine atherosclerosis and rat carotid balloon injury models respectively.

Integrating Chappell et al.[6] observations with these classic SMC growth fraction studies[8, 9] suggests that a larger proportion of medial SMC may undergo proliferation but that only certain clonal populations survive a robust process of clonal selection. Indeed, several plausible alternative or complementary mechanisms could explain the lesion oligoclonality. *First*, the overgrowth of a few hyperproliferative cells might simply out-compete other clones or cellular populations. *Second*, the expansion of a particular clone within the lesion may be due to, not only its unique ability to proliferate within the media, but also to its capacity to migrate into lesions and survive the lesion environment. For example, certain SMC clones might be differentially susceptible to apoptosis, efferocytosis and clearance by differential expression of cell cycle regulators like CDKN2B[10], and/or “eat me” and ”don’t eat me” ligands like CD47[11], as shown beautifully in a recent *Nature* paper by Nick Leeper’s lab. That is, a clone that became resistant to apoptosis and clearance would become dominant within lesions. *Third*, a few medial SMC clones might become dominant by inhibiting the clonal expansion capacity of surrounding cells. This mechanism implies that a dominant clone repress the ability of cells within close vicinity to proliferate and/or migrate within the lesion by cell-cell interaction or secreted factors. The possibility of effects on migration is of particular interest and relevance with respect to the involvement of neuroguidance molecules like Netrin-1[12] that have been shown to play a critical role in lesion pathogenesis by regulating SMC and macrophages chemoattraction and chemorepulsion. Furthermore, such a mechanism of paracrine or juxtacrine inhibition of clonal expansion may serve as an evolutionary protective mechanism to prevent impairment

of the contractile properties of an injured or diseased blood vessel ensuring that only a subset of SMC dedifferentiate.

The fact that only a few medial cells behave drastically differently compared with surrounding medial cells while they are probably exposed to similar modifications of environmental cues (e.g. hypercholesterolemia, blood flow perturbation) raises the question of the mechanisms priming these cells to expand or become dominant. One possibility is that a given SMC clone becomes dominant by genetic and/or epigenetic priming. For example, recent studies have shown that occurrence of somatic mutations in a small subset of myeloid cells causes clonal expansion and is correlated with an increased cumulative incidence of cardiovascular events in humans[13]. Assuming similar mechanisms are operative in SMC, somatic mutations in even a very small number of medial SMC could have profound effects on lesion pathogenesis if the mutations confer a proliferation or survival advantage. We also anticipate that epigenetic reprogramming plays a role in SMC clonal expansion and/or selection. Indeed, we have recently shown that epigenetic reprogramming, namely DNA hydroxymethylation, plays a critical role in activation of the pluripotency factor Oct4 in atherosclerosis. Importantly, SMC-specific KO of Oct4 leads to a marked depletion of SMC within advanced atherosclerotic lesions likely due to impairment of migratory capacity of the medial SMC. Although the frequency of DNA hydroxymethylation of the Oct4 gene among with the medial SMC population is unknown, this or similar mechanisms may be involved in the clonality of lesion SMC.

In summary, the study by Chappell et al.[6] is the first study to clearly show that SMC population of neointima following vascular injury or experimental atherosclerosis is derived from a subset of $Myh11^{+}$ medial SMC. These results are very important and relevant in our understanding of the participation of differentiated medial SMC to neointima formation. However, further mechanistic studies are required to clarify by which mechanisms certain SMC clones become dominant. Indeed, investigation of the mechanisms controlling each aspect of SMC behavior (clonality, phenotypic modulation and transitions) during atherosclerosis development and progression could give rise to novel personalized therapeutic strategies aimed at increasing the stability of vulnerable SMC-poor lesions by selectively promoting the expansion of “beneficial” plaque stabilizing SMC clones.

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