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Strength in Numbers: Cardiac Fibroblast Clustering and Myocardial Remodeling

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Short title: Fibroblast clustering and cardiac remodeling

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In the normal heart, cardiac fibroblasts are localized in the interstitium between cardiomyocytes where they are relatively quiescent and play a supportive role in cardiac homeostasis. When the heart is stressed or injured, fibroblasts become activated and migrate to damaged regions where they proliferate and facilitate wound healing and repair.[1] Cardiac fibroblasts exhibit a high level of plasticity and are able to adopt a number of different phenotypes during the remodeling process, including differentiation into myofibroblasts. The topology of fibroblasts in the normal heart, in which cells are largely separated from one another, is therefore very different from the remodeling heart, in which clustering of myofibroblasts occurs due to a combination of cell migration, proliferation and differentiation.

The article published by Yu et al[2] in the current issue of *Circulation Research* proposes that simple topological rearrangement of cardiac fibroblasts from a 2D environment to a 3D spherical cluster is sufficient to induce chromatin remodeling and gene expression changes that correlate with those observed in the remodeling heart *in vivo*, and importantly are associated with indices of adverse cardiac remodeling (see **Figure**).

Accurate identification of cardiac fibroblasts has been confounded by a lack of cell-specific markers for this cell type. Much of our historical knowledge on the role of cardiac fibroblasts *in vivo* has used markers such as vimentin, Thy1 and FSP1; but these proteins are also expressed by a range of other cardiac cell types.[3] The advent of fluorescent lineage-tracing technologies has transformed our understanding of the role and fate of cardiac fibroblasts in cardiac development, physiology and pathophysiology. Several mouse lines have been generated that enable fibroblasts and myofibroblasts to be accurately traced over time in the remodeling heart.[4,5] These studies have provided important new insights into the localization, phenotypic conversion and importance of fibroblasts in the remodeling heart.

The article by Yu et al[2] used 2D and 3D *in vitro* cell culture techniques, combined with in-depth profiling of gene expression (RNA-Seq) and chromatin accessibility (ATAC-Seq), to demonstrate that simple topological rearrangement of cardiac fibroblasts into 3D spherical clusters can induce changes akin to those observed in the remodeling heart. The differential gene expression signatures between 2D and 3D cultures were reversible, reflecting the remarkable plasticity of this cell type. Important controls confirmed that these changes were not simply due to differences in tension/stiffness of the environment between 2D cultures and 3D aggregates. Gene ontology analysis highlighted patterns of genes that were down-regulated (DNA replication, chromosomal condensation/segregation, cytokinesis) and up-regulated (ECM metabolism/proteolysis, surface proteins, chemotaxis and immune response) in the 3D fibroblast clusters compared with the 2D cultures. Follow-up studies confirmed that 3D-aggregated cells exhibited markedly reduced proliferation, reduced expression of contractile proteins (e.g. the myofibroblast marker αSMA), reduced collagen expression, increased MMP expression and increased polarity. The latter point suggests that 3D fibroblast aggregates have features of aligned topology, as observed for myofibroblasts in the infarct border zone.[6]

The *in vivo* relevance of the 3D fibroblast clusters was investigated by comparing *in vitro* RNA-Seq gene expression signatures with those of the remodeling heart (using a data reduction method involving generation of principle component eigengenes). The authors used two different models of cardiac remodeling; an isoproterenol infusion heart failure model and a cryo-injury model to mimic myocardial infarction (MI). For the isoproterenol model, an extensive study was undertaken using 96 genetically diverse mouse strains (Hybrid Mouse Diversity Panel), an approach designed to enable genome-wide association analysis of phenotypic traits. Data were collected on the severity of cardiac hypertrophy and heart failure, and ventricular RNA extracted for gene expression analysis to compare with 2D/3D cultures. Significant correlations were observed between the eigengene 3D gene signatures and adverse cardiac indices (e.g. hypertrophy, dilatation). It is worth noting that ventricular RNA from isoproterenol-infused mice was extracted from intact tissue rather than specifically from fibroblasts so would also contain RNA from other cardiac cell populations. Nevertheless, the results still provided a strong correlation between the fibroblast 3D signature and adverse cardiac parameters. For the cryo-injury model, the authors used

fibroblast lineage-tracing reporters to identify aggregated fibroblasts 7 days after injury and correlated this with expression of some of the 3D-upregulated proteins (e.g. MMP-11). Optical clearing of the hearts combined with confocal microscopy also highlighted association of aggregated fibroblasts with 3D-upregulated markers. Whilst these confirmatory experiments are generally supportive of the complex *in vitro* data, their limited focus on a very small number of selected targets did not capture the true importance of the gene signatures.

Cardiac fibroblasts play a critical role in modulating cardiomyocyte hypertrophy through paracrine secretion of growth factors and other secreted molecules.[7,8] The authors used live cell interferometry to show that neonatal rat ventricular myocytes underwent hypertrophy in response to conditioned media collected from cardiac fibroblast cultures, and that importantly the hypertrophic effect was larger in response to media from 3D cultures compared with 2D cultures.[2] Thus, the secretome from aggregating cardiac fibroblasts may directly contribute to cardiac hypertrophy after myocardial injury.

The study by Yu et al[2] raises several important questions, the most fundamental being: what is the *in vivo* cardiac fibroblast phenotype that is represented by the *in vitro* 3D fibroblast aggregates? Fibroblasts are known to adopt a range of phenotypes at various stages after MI.[3] Recent experiments by Fu et al[9] used multiple fibroblast lineage-tracing Cre-expressing mouse lines, combined with mRNA profiling and confocal immunohistochemistry, to more precisely define fibroblast differentiation states in the post-MI heart. These were described as 'quiescent' (pre-injury), 'activated' (day 2-4 post-MI), 'myofibroblast' (day 4-7 post-MI) and 'matrifibrocyte' (day >10 post-MI); the latter being a highly differentiated, non-proliferating cell with a tendon-like gene signature important for maintaining scar integrity. How the 3D aggregates of cardiac fibroblasts described in the article by Yu et al[2] correlate with these phenotypes is not immediately clear. The 3D gene signature included reduced αSMA, increased MMPs, increased inflammatory factors and reduced fibrotic factors. These hallmarks are similar to those observed in cardiac fibroblasts following stimulation with proinflammatory cytokines,[10] representing the early inflammatory phase of post-MI remodeling (days 1-2) prior to fibroblast activation.[3] However, the correlation in gene signatures between 3D-clustered cells and remodeling hearts (3 weeks isoproterenol infusion or 7 days after cryo-injury) indicate the 3D signature represents later phases of remodeling at a time when myofibroblasts are prevalent. In this regard, the decrease in αSMA expression observed between 2D and 3D cultures is perplexing as αSMA is characteristic of the myofibroblast phenotype. It would certainly be interesting to know whether the gene signature of the 3D clustered fibroblasts (reduced proliferation, reduced αSMA, reduced ECM synthesis) correlates with that of the newly described matrifibrocytes[9] that are present in mature scars, as these phenotypes, at least on first impression, appear similar.

MMP-11 (stromelysin-3) was one of the most highly up-regulated genes in 3D fibroblast clusters, and was studied further *in vivo*.[2] The matrix metalloproteinases are a large family of zinc-dependent endopeptidases that together can degrade all components of the ECM. Cardiac fibroblasts express several different MMPs that are important for remodeling the cardiac ECM after injury.[11] MMP-11 is unusual in that it is secreted in an active form (unlike other MMPs that are secreted as zymogens) and acts primarily on non-ECM components. This MMP has not been well-studied in the heart, although there is evidence that its ventricular expression is differentially regulated by hypoxia.[12] MMP-11 has been more extensively studied in the cancer field; it is elevated in solid tumor biopsies and in the sera of cancer patients, and plays a role in development and progression of solid tumors.[13] Obvious parallels can be drawn between the aggregation of cancer cells and clustering of cardiac fibroblasts in this respect. Future studies with cardiac fibroblast-specific deletion of MMP-11 would be helpful in determining whether this protease is truly an important player in regulating fibroblast function and cardiac remodeling.

In summary, the study by Yu et al[2] presents interesting new data using a range of novel techniques that suggest that cardiac fibroblast aggregation *per se* is sufficient to drive

phenotypic changes that contribute to the cardiac remodeling process. Further analysis of the 3D gene signatures with respect to known phenotypes of cardiac fibroblasts in the remodeling heart will be important to decipher the true importance of cell clustering in regulating fibroblast differentiation and remodeling. Such analyses may in turn identify novel targets for developing therapeutic agents to reduce adverse cardiac remodeling after myocardial injury.

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Disclosures
None.

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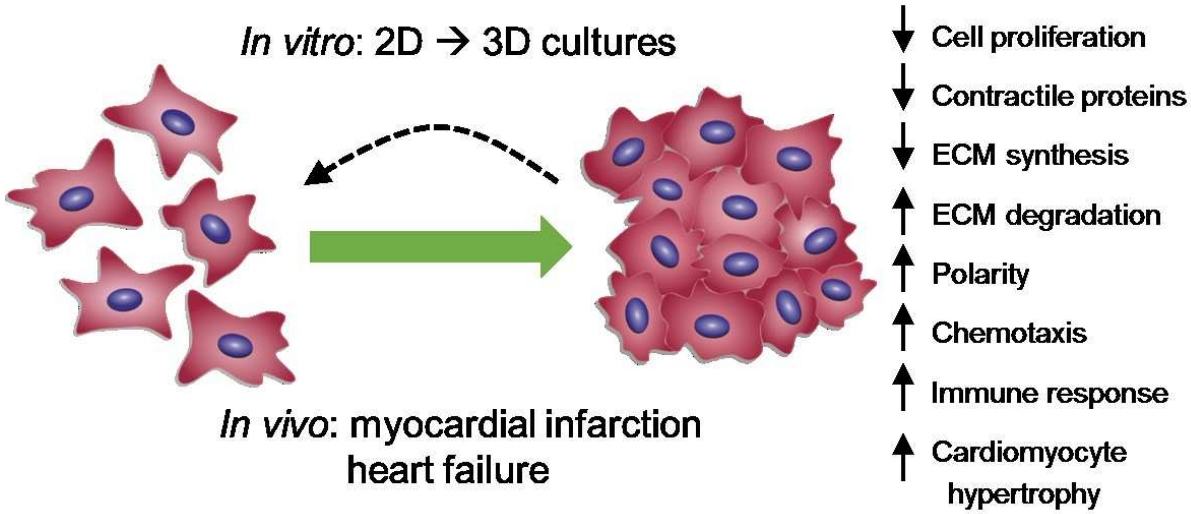

Figure. Role of cardiac fibroblast aggregation on phenotype. 3D clustering of cardiac fibroblasts *in vitro* drives gene expression changes associated with reduced proliferation, reduced ECM synthesis and reduced expression of contractile proteins. Conversely, gene networks involved with ECM degradation, polarity, chemotaxis and immune response are increased. 3D-clustered cardiac fibroblasts are more potent inducers of cardiomyocyte hypertrophy than 2D cultures. These features are reversible *in vitro* (dashed arrow). Overlapping gene signatures were observed in remodeled cardiac tissue from *in vivo* models of heart failure and myocardial infarction. Moreover, 3D gene signatures were associated with indices of adverse remodeling.