On the cellular origin of cardiosphere-derived cells (CDCs)

Eduardo Marbán¹ · Ke Liao¹

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Over the past 17 years, cardiosphere-derived cells (CDCs) have been developed as a therapeutic candidate for regenerative therapy [23, 30]. These cardiac stromal cells (which are entirely distinct from discredited c-kit-positive cells [7, 36]) exhibit multilineage potential and clonogenicity [10, 30], thus qualifying as progenitors, but work primarily through indirect mechanisms [9, 14]. At least 70 independent labs worldwide have published on CDCs (see, e.g. [1, 11, 27, 31, 32, 39]). Meanwhile, ten clinical trials, seven in the USA and three independently performed in Japan, have investigated CDCs for cell therapy. The first, an NIH-funded trial called CADUCEUS [19, 21], used intracoronary autologous CDCs in patients with mild heart failure with reduced ejection fraction (HFrEF) following myocardial infarction (MI). The Japanese trials have likewise used autologous CDCs, with a focus on hypoplastic left ventricle (LV) or dilated cardiomyopathy [31]. Subsequent clinical trials in the USA have used allogeneic CDCs, which were found to produce equivalent benefits but are more realistic as clinical products [20, 23]. These include ALLSTAR [18] (post-MI patients with mild HFrEF; NCT01458405), DYNAMIC [6] (advanced HFrEF; NCT02293603), and HOPE-Duchenne [33] and HOPE-2 [25] (for muscular dystrophy and its associated cardiomyopathy; NCT02485938 and NCT03406780). Those trials, now completed, have verified not only are CDCs safe, but also they may have disease-modifying bioactivity in humans. Mechanistically, the prevailing concept of CDC efficacy centers on the “paracrine hypothesis”, which posits transplanted cells produce salutary soluble factors [13]. Benefits of CDCs and their secreted paracrine factors include anti-fibrotic effects [34,

35], anti-apoptotic effects on myocytes [8, 16], angiogenesis [9, 34], modulation of inflammatory processes and oxidative stress [3, 34], and promotion of cardiomyocyte cell cycle re-entry [22]. CDC-secreted exosomes, CDC_{EXO} (which are a class of extracellular vesicles), mediate the major benefits of CDCs via cell–cell transfer of therapeutic payloads [2, 14, 29]. Analysis of CDC_{EXO} payload using RNA sequencing (RNA seq) and proteomics has revealed numerous species of noncoding RNA (ncRNA) and proteins [5, 14], some of which are known to be bioactive and others which have yet to be functionally characterized.

In the beleaguered field of cardiac cell therapy, CDCs are rare in their continued viability along the clinical development pathway [24]. Despite the extensive, and growing, interest in this cell type, relatively little is known about their cellular origin. We do know that CDCs are intrinsic cardiac cells; they do not arise from hematogenous seeding [38]. CDCs are uniformly CD105 positive but, unlike fibroblasts, are low in CD90 (an antigen whose presence correlates negatively with regenerative potency [7, 12]). Moreover, genetic lineage tracing in mice has revealed that CDCs do not originate from cardiomyocytes [40]. Beyond that, however, the origin of CDCs remains mysterious. Presumably, CDCs arise from a cell type present in intact myocardial stroma, but which proliferates in primary culture during the cardiosphere formation process.

Correctly recognizing the knowledge deficit as to CDC origin, Doppler and colleagues [15] have tackled this question using contemporary cell phenotyping approaches. CDCs are compared to non-myocyte cell types (cardiac fibroblasts [CFs], smooth muscle cells [SMCs], endothelial cells [ECs], and adipose tissue derived fibroblasts [AFs]). Single cell-RNAseq was used to gauge the transcriptional similarities and differences among these various cell types. CDCs emerge as a unique cell type, distinct from any other. A striking finding, one of the most impactful in the paper, is that genes coding for the chemokine ligand CXCL6 and the cytokine IL-1 β were highly expressed in CDCs but not in any of the comparator cell types, suggesting CXCL6 and IL-1 β could serve as novel discriminating markers specific

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✉ Eduardo Marbán
eduardo.marban@cshs.org

¹ Cedars-Sinai Medical Center, Smidt Heart Institute, 127 South San Vicente Boulevard, AHSP A3600, Los Angeles, CA 90048, USA

to CDCs. Additionally, mitochondrial genes were richly expressed in CDCs, but not in CFs, SMCs or ECs. Using bioinformatics approaches [37] to compare these cell populations, CDCs were found to be unique, albeit with some similarities to CFs, SMCs and ECs.

The authors further push the notion of similarity to CFs, and even go so far as to speculate that CDCs might originate from CFs. However, there is neither good evidence to support this claim, nor are the methods capable of definitively testing this claim. The major piece of evidence cited for the conjecture that CDCs arise from CFs is Fig. 3, panels D and F. Panel D shows pseudotime trajectories. Such analysis is typically undertaken not to compare diverse cell types, but rather to infer temporal gene expression changes within a single cell type studied under various conditions (e.g., as might have been done to compare old and young CDCs, or the transition from a precancerous state to a cancerous one in a given line). Here, the analysis has been pushed to situations where it was never meant to be applied. To quote from a recent review in the *Journal of Immunology* [26]: “The analytical approach called pseudotime analysis *assumes a continuum of cellular states* and aims to reveal cellular progression by inferring cellular trajectories. This type of analysis is particularly useful for studying cellular differentiation or disease progress.” Here, the authors ASSUME that CDCs and CFs comprise a continuum of cellular states, forcing the analysis to reach biologically dubious conclusions. The algorithms will create trajectories no matter what they are fed; it is up to the biologists to stipulate what comparisons make sense. In Fig. 3F, Velocyto software is used to generate RNA velocity analyses. Once again here, the fundamental question seems not to adhere to the situation being analyzed. Quoting Bergen et al.[4]: “Velocyto estimates velocities under the assumptions... that (1), on the gene level, the full splicing dynamics with transcriptional induction, repression and steady-state mRNA levels are captured; and (2), on the cellular level, all genes share a common splicing rate. These assumptions are often violated, in particular when a *population comprises multiple heterogeneous subpopulations with different kinetics*.” Clearly, here the cluster analysis, and the very fact of how the cells were isolated, testify to the presence of “multiple heterogeneous subpopulations”, undermining the RNA velocity analysis. Thus, it is unclear what, if anything, can be gleaned from Fig. 3D, F, but certainly it cannot be concluded that CDCs are derived from CFs. Genetic lineage analysis, which the authors did not attempt, would have been required to draw firm conclusions about cell origin.

The authors further contrast CDCs to what they call “cardiac progenitor cells” derived from human iPS cells, and found no overlap between these two cell types by cluster analysis. Once again, however, the conclusions are overstated. The “cardiac progenitor” differentiation

Fig. 1 Schematic diagram demonstrating molecular identity of CDCs and effects of CDC-EVs. Cardiosphere-derived cells (CDCs), a kind of cardiac stromal/progenitor cell, obtained from heart biopsy after a series of primary culture steps. They are of intrinsic cardiac origin with uniform expression of CD105 (endoglin), but are negative for the pan-hematopoietic marker (CD45). New findings from group of Doppler reveal that CXCL6 and IL1- β are specific transcriptomic markers for CDCs. Secreted extracellular vesicles mediate the disease-modifying bioactivity of CDCs

protocol used in this paper actually produces *cardiomyocyte* precursors [17, 28]. The contrast with CDCs is thus not surprising, as it is already known, from lineage tracing using MerCreMer-Z/EG transgenic mice, that CDCs do not originate from cardiomyocytes [10].

Finally, the functional testing of CDCs here, while not extensive, yielded anemic results. As aforementioned, CD90, also known as thymocyte differentiation antigen 1, is negatively associated with CDC potency: the higher the CD90 fraction, the lower the observed preclinical benefits [7, 12]. The CDCs used here contain high proportions of CD90+ cells, rationalizing their limited efficacy.

So what can be reliably concluded from the extensive work by Doppler et al., in the context of what was already known about CDCs? As summarized in Fig. 1, CDCs are a unique cell type in terms of their antigenic (high CD105, negative for CD45) and transcriptomic profiles (high CXCL6, IL1 β). They are of intrinsic cardiac origin. While the present study has added evidence for the uniqueness of CDCs, we are no further along in identifying their precise cellular origin, or in vivo correlates, than we were before.

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Declarations

Conflict of interest E. Marbán holds the Mark S. Siegel Family Distinguished Chair of the Cedars-Sinai Medical Center. E. Marbán owns founder's equity in Capricor Therapeutics. The other author report no conflicts.

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