Review

Ex uno, plures—From One Tissue to Many Cells: A Review of Single-Cell Transcriptomics in Cardiovascular Biology

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Abstract: Recent technological advances have revolutionized the study of tissue biology and garnered a greater appreciation for tissue complexity. In order to understand cardiac development, heart tissue homeostasis, and the effects of stress and injury on the cardiovascular system, it is essential to characterize the heart at high cellular resolution. Single-cell profiling provides a more precise definition of tissue composition, cell differentiation trajectories, and intercellular communication, compared to classical bulk approaches. Here, we aim to review how recent single-cell multi-omic studies have changed our understanding of cell dynamics during cardiac development, and in the healthy and diseased adult myocardium.

Keywords: heart; cardiac; single-cell; scRNAseq; differentiation; development; injury; regeneration

1. Introduction

The heart is our first functional organ. It starts beating very early in development, around E8.5 in mouse or 5–6 weeks of gestation in humans, and it beats about 3 billion times in an average human lifetime, pumping blood throughout the body to provide oxygen and nutrients while removing waste. This hard-working fluid pump function is achieved thanks to cardiomyocytes (CMs), which are highly specialized striated muscle cells, as well as a network of interstitial cells from vascular, nervous, immune, and mesenchymal/stromal lineages. Interstitial cells are responsible for building the proper 3D scaffold of the heart, ensuring proper CM alignment and synchronous beating. In response to injury, interstitial cells orchestrate the reparative response, leading to the scar formation, in order to compensate for the poor proliferative and regenerative capacity of CMs in adult mammals [1].

Gene expression studies are an accessible and cost-effective method for querying the molecular state of the heart during development, at homeostasis, or in response to insult. Until recently, these studies have largely been conducted either on cell populations enriched by cell sorting or on bulk cardiac tissue. Although both approaches have provided extremely valuable information, each is hampered by significant drawbacks. First, the main cellular constituents in cardiac physiology and disease have been defined based on the expression (or the lack thereof) of a limited number of surface antigens; thus, sorted cell populations may represent only a subset or a particular state within the cell population of interest [1]. Second, bulk tissue transcriptomics is biased toward the most prevalent cell types in the tissue, is confounded by compositional heterogeneity between samples, and may not represent the full spectrum of states within a cell population.

Single-cell RNA sequencing (scRNAseq) has emerged as a high-resolution alternative to bulk RNAseq and has gained popularity as a cardiovascular research tool (Figure 1A). Recent advances in scRNAseq technologies have enabled the profiling of individual cells in...
an unbiased fashion. Emerging methods for the analysis of scRNAseq data typically utilize a high-dimensional gene signature rather than any pre-selected list of canonical markers. These techniques have revealed significant cellular heterogeneity, paracrine inter-cellular communication among distinct cardiac cell populations, transcriptional kinships, potential intermediate states, and putative cellular differentiation trajectories.

Figure 1. Overview of the exponential growth in single-cell transcriptomic studies from 2015 to 2020. (A) The number of published cardiovascular studies using single-cell transcriptomics per year. (B) The number of cells sequenced in each study. Studies in which the number of sampled cells could not be determined were omitted.

The first attempts to sequence RNA from single cells were published almost 30 years ago and involved the manual microinjection of sequencing reagents into each dissociated cell [2–4]. Initial studies were limited to the analysis of a few genes by PCR. The development of integrated fluidic circuits (IFC) allowed for the quantification of the expression of multiple genes from the same cell, by reducing the reaction volume (Fluidigm
Dynamic Arrays [5,6]). By optimizing cDNA library preparation, it became possible to capture whole-transcriptomes from single cells [7]. As of today, several scRNAseq protocols have been reported. One primary differentiating feature of these protocols is the method of cell isolation. These include methods that use nanofluidic traps (Fluidigm C1 IFC); the distribution of cells in wells by manual dilution, robotic dispersion, or FACS sorting (ICell8 Takara, Smart-seq2 [8], SORT-seq [9], Microwell-Seq [10], sci-RNA-seq3 [11]); and droplet-based systems (Drop-seq [12], sNucDrop-seq [13], InDrop [14]; Chromium Single Cells-10xGenomics [15]). The introduction of methods for automatic cell capture, lysis, and cDNA synthesis, together with the use of unique molecular identifiers (UMIs) [16] and molecular-based barcoding to multiplex cDNA amplification have contributed to plummeting per-cell costs, as evidenced by the exponentially increasing number of analyzed cells per study (Figure 1B). Compared to plate-based full-length sequencing [8], tag-based methods [9–16] detect fewer genes and capture only the 3′ or 5′ end of each transcript; however, the presence of UMIs improves gene-level quantification and mitigates biases in cDNA amplification. Moreover, new barcoding systems are allowing researchers to combine transcriptomic profiles with additional information from individual cells. For example, using polyadenylated DNA barcodes conjugated to antibodies, it is possible to estimate the abundance of specific surface antigens [17,18]. For an in-depth analysis of the history of scRNAseq and the comparison among different methods, we refer to [19–22].

Here, we review how single-cell technologies have impacted the cardiovascular field by allowing for high-resolution mapping of the heart at different stages of development and in pathological conditions, providing new insights on intercellular communication, cellular dynamic changes, and regulatory networks, as well as highlighting new potential therapeutic targets and prognostic biomarkers for patient stratification. Finally, we address the current limitations and future directions of the single-cell revolution.

2. Cardiac scRNAseq during Embryonic and Postnatal Development in Physiology and Disease

In the cardiac context, developmental biology and stem cell biology were the first fields to benefit from emerging single-cell transcriptomic techniques, which were used to define the cell fate and lineage trajectories in the developing heart and in vitro differentiating cells (Table 1). Two studies in 2015 adopted manual cell lysis and cDNA preparation combined with targeted single-cell qPCR (sc-qPCR) to analyze the early stages of cardiovascular commitment. Li et al. [23] first defined a panel of genes to distinguish all the main cardiovascular lineages, and they applied it to compare embryo- and mouse embryonic stem cell (mESC)-derived cardiac progenitor (CPs) and CMs at different stages; then, using time-lapse microscopy and multiplex sc-qPCR, they showed that while mESC-derived Nkx2.5+ CPs are bipotent and can become either CMs or smooth muscle cells (SMCs), Nkx2.5+ CPs in the embryos include two distinct unipotent subsets that differentiate either into endothelial cells (ECs) or CMs. Kokkinopoulos et al. [24] used a similar approach to profile about a thousand cells obtained from microdissected heart-forming regions from the early allantoic bud stage (EB, E6) to the early headfold stage (EHF, E8). After classifying the single-cell cDNA preparation chronologically, based on the expression of key markers, they selected 12 cells across four stages for deep sequencing, and the authors conclude that in the First Heart Field (FHF), Tbx5+ CPs exist transiently in the progenitor stage and rapidly differentiate post-activation of Nkx2.5 expression, after the EHF stage. One year later, two landmark papers provided the first transcriptomic maps of the developing hearts on multiple time points, using a semi-automated system, the Fluidigm C1 Integrated fluidic circuits (IFC), which allows for the capture, lysis, and retrotranscription of up to 96 single cells simultaneously [25,26]. The first study [25] profiled an unbiased selection of about 1200 cells from seven time points spanning from the early heart tube stage to the post-natal heart (E9.5, E11.5, E14.5, E18.5, P0, P3, and p21). The authors used this dataset as a reference to identify the developmental ages of mouse and human ESCs and to investigate the effect of the heterozygous mutations in Nkx2.5 on lineage-specific differentiation. The second study [26] focused on three time points (E8.5, E9.5, E10.5),
carefully dissecting two, seven, and nine regions, respectively. Combining transcriptomic and spatial information, they generated an algorithm to predict the anatomic origin of single CMs and the distribution of SHF Isl1+ derived cells, as well as to detect blocks in differentiation based on transcriptomic data. For example, when applied to Nkx2-5−/− CMs from E9.5 embryos, the algorithm revealed that a persistent atrial-like phenotype underlies the lack of ventricle development. The same technology has subsequently been used to selectively profile αMHC+ CMs in early and late development and post-natal hearts [27].

Additional studies have used the combination of lineage tracing, tissue microdissection, and scRNAseq to investigate the differentiation trajectory of CPs from the FHF and SHF [28–30]. Jia et al. [28] isolated CPs from Nkx2-5-emGFP transgenic and Isl1αGFP+ knockin embryos at E7.5, E8.5, and E9.5 and through scRNAseq, using Fluidigm C1 IFCs, and snATACseq (single nucleus assay for transposase-accessible chromatin with sequencing), they confirmed that Isl1+ CPs pass through an intermediate state before separating into different fates, whereas Nkx2-5+ CPs are unidirectionally committed to CM differentiation. Similarly, in a later study [30], the authors used Isl1Cre::Rosa26tdTomato/+ and Nkx2-5Cre::Rosa26tdTomato/+ embryos to isolate cells derived from the two lineages at E8.25, 8.75, 9.25, and they performed scRNAseq by Smart-seq2 [8] on manually isolated cells. They confirmed the previously identified pattern of differentiation of FHF and SHF CPs, and through ligand–receptor analysis, the authors suggest the Mif–Cxcr2 pair as a possible mediator of the SHF CPs chemotaxis guided by CMs, which is a result that was confirmed by pharmacological inhibition and genetic knockouts of both Cxcr2/Cxcr4. Similarly, lineage tracing combined with Smart-seq2 has been used to analyze the arterial specification in the sinus venosus [31] and epicardial development in zebrafish [32].

Genetic knockouts, lineage tracing, and scRNAseq have been used in combination to define the early stage of cardiovascular lineage segregation from Mesp1+ mesodermal precursors [33], the role of the Hippo pathway [34], Pitx2 [35], and pbx4 [36] in development, the effect of Hand2os1 lncRNA [37], and full microRNA KO [38]. The first study, similarly to what was described earlier [39,40], showed that Mesp1+ cells commit very early to different cardiovascular lineages, and the transcriptomic profiling at a single-cell level allowed for the identification of early pathways, determining the commitment to different cell fates, such as Notch1 for the endocardium. Furthermore, by comparing Mesp1 wt and null cells at early gastrulation (E6.75), the authors showed that Mesp1 expression is required to exit the pluripotent state.

The second study used epicardial specific conditional knockout of the kinases Lats1/2 to interrogate the role of Hippo signaling in the commitment and differentiation of epicardial-derived cells. The authors profiled cells from wt and KO embryos at E13.5 and 14.5 before the appearance of cardiac phenotypes in the mutant using a high-throughput droplet-based system, Drop-seq [12], and they observed that mutant cells failed to differentiate in fibroblasts, maintaining an intermediate state with the expression of both epicardial and fibroblast markers. Additional pharmacological and genetic validation confirmed that an absence of Lats1/2 increased the nuclear localization of Yap, which inhibited fibroblast differentiation.

The third study [35] used scRNAseq in murine E10.5 and E13.5 wt and null embryos to analyze the molecular and cellular consequences of Pitx2 disruption. Pitx2 is a homeobox transcription factor, with pleiotropic functions during development. It is involved in OFT and valve development, left-right specification of the atria, and its loss has been implicated in human atrial fibrillation [41,42]. Consistent with previous work, the authors noted an altered cardiac cell composition and transcriptional changes in SHF progenitors that could lead to the prevalent differentiation in RA and sinoatrial node CMs.

A fourth study focused on Pbx4, a transcription factor similarly involved in SHF specification in zebrafish [36]. Single-cell transcriptomic analysis of Pbx4-depleted SHF progenitors showed a lack of heterogeneity and increased proliferation, which was in line with the cardiac dilation phenotype, and supporting its crucial role in the definition of the anterior and posterior progenitor fields that contribute to OFT and pharyngeal
arches, respectively. In a fifth study [37], the authors showed that lncRNA Hand2os1 strictly
regulates Hand2 expression in mouse; its full knockout caused a slight increase of Hand2 in
all cell types, which was sufficient to induce changes in cell composition and cell-specific
transcriptome, leading to morphological and functional abnormalities. In a sixth study [38],
full microRNA KO in CMs was achieved by conditionally deleting the microprocessor
Dgcr8 in Mesp1+ cells. As a result, the heart appeared dilated and the CMs upregulated
vascular genes, demonstrating that microRNAs are required to suppress the angiogenesis
program and allow CM differentiation.

Unbiased profiling of microdissected regions and paired-scRNAseq by Smart-seq2
have been used to define early mesoderm patterning during gastrulation (E6.5–7) [43],
and more recently, to generate a highly time- and space-resolved profile of the mouse
anterior cardiac region from the cardiac crescent to the linear tube stage [44]. For this latter
study, the author collected samples at six time points, 12 h apart (E7.75–8.25), identified
six cardiac clusters at different levels of differentiation, and assigned them to specific
locations of the developing heart using scRNAseq data from four dissected regions of the
cardiac mesoderm as a spatial reference. Based on gene expression and localization, the
sub-populations were attributed to SHF or FHF, and differentiation trajectories, inferred
on transcriptomic statuses, were validated by lineage tracing combined with single-cell
resolution microscopy and in situ hybridization chain reaction. These analyses led to the
identification of an extra progenitor field, anterior to the cardiac crescent, which was named
the juxta-cardiac field (JFC). These Nkx2-5− Hand1+ Mab21l2+ cells are derived from Mesp1+
progenitors and contribute to both the proepicardium and the myocardium.

Microdissection and high-throughput scRNAseq, using the 10x Chromium technology,
enabled the characterization of the murine cardiac outflow tract development in early,
middle, and late stages of remodeling and septation (47-, 49-, 51- pairs of somites, E11-
12) [45], as well as of the wild-type murine conduction system (at E16.5) [46], providing
reference transcriptomic maps to compare normal and altered conditions.

A few studies have aimed to define atlases of the full cardiac embryonic development.
In the same year, one study profiled about 4000 cells from different stages of human fetal
heart development [47], and another defined a Mouse Organogenic Cell Atlas (MOCA),
analyzing over 2,000,000 total cells from murine embryos from five different stages, in-
cluding over 7000 cells of the cardiac lineage [11]. In the first study, the authors isolated
cells from various regions of fetal hearts in early (5–7 weeks), mid (9–17 weeks), and
end stage (20–25 weeks) of gestation. Cells were manually picked, lysed, and the RNA
was retrotranscribed using a modified version of STRT-Seq (Single-Cell Tagged Reverse
Transcription Sequencing) [16]. The authors identified four main cell clusters, defining
their spatial distribution and progressive transcriptomic changes during development. Fur-
thermore, the authors compared these data with previously published mouse embryonic
datasets [26,34] to identify correspondent stages and highlight the common and unique
markers of each cell type. For the second study, the authors used a different plate-based
system, sci-RNA-seq3 [48], that utilizes combinatorial indexing to profile millions of cells
in the same experiment. In this particular study, nuclei were isolated from snap-frozen
murine embryos (E9.5, 10.5, 11.5, 12.5, and 13.5 embryos), profiling enough cells to cover
3–80% of a full embryo per stage. Despite shallow sequencing allowing the detection of
only a few hundred transcripts per cell, the authors were able to identify over 500 cell
subtypes and 56 developmental trajectories, which can be publicly explored online [49].

More recently, researchers have pioneered unbiased Spatial Transcriptomics [50] and
combined it with in situ sequencing by single molecule fluorescence in situ hybridization
(smFISH), and scRNAseq to recreate organ-wide cell type/gene expression maps of the
developing human heart in three main stages: 4.5–5, 6.5–7, and 9 weeks post conception [51].
These data can be explored both in 2D and 3D on a public web platform [52].

One potential use of these publicly available atlases is to provide a baseline framework
for comparison to congenital diseases and abnormal conditions. At least three studies in the
past three years have used scRNAseq to investigate congenital heart diseases. Hu et al. [53]
used a droplet microfluidic-based snRNA-seq method (sNucDrop-seq [13]) to unbiasedly profile cells from post-natal hearts of wild-type (P6 and p10) and ERRα/γ knockout mice (p10) [54], creating a model of pediatric mitochondrial cardiomyopathy. They observed cell-specific gene expression changes, with CMs, fibroblasts, and ECs being the most affected cell types. Most of the cells presented a downregulation of genes related to oxidative phosphorylation, and an upregulation of fibrosis genes was observed also in non-fibroblasts. Furthermore, single-cell transcriptomic data revealed that the expression of Gdf15, a cardiac-produced hormone and marker of cardiac dysfunction, was regulated by distinct cell-type-specific networks. More recently, two studies have used 10x Chromium technologies to investigate different models of congenital heart defects [55–58]. The first study [55] profiled the cardiogenic region of wild-type versus Hand2 null mouse embryos over three phases of cardiac development (E7.75, E8.5, E9.25). The Hand2 deletion causes embryonic lethality by E10.5, in the absence of right ventricle development [59]. Temporal transcriptomic analysis revealed that RV precursors were indeed specified but failed to properly migrate and differentiate, while OFT specification was impaired, thus providing new insights on the cellular mechanisms of the altered development. A case of autoimmune-mediated congenital heart block (CHB) in a 21-week-old human fetus has been analyzed by profiling cardiac cells from the affected fetus and cells from control fetuses at 19–22 weeks of gestation [57]. CHB affects mid-gestation normal developing fetuses that have been exposed to maternal autoantibodies. Differential gene expression revealed an increase and heterogeneous IFN response in different cell types of the affected heart and the expression of ECM genes, which is in line with the observed fibrosis in the AV node.

In summary, single-cell transcriptomics has facilitated advances in several areas of developmental biology: the derivation of spatial and temporal maps of embryonic cardiac morphogenesis, the identification of new progenitor fields without the restraints of known marker genes, and the inference of cell–cell interactions and differentiation trajectories, which can then be validated with classical approaches such as lineage tracing or gene knockdown. These high-resolution road maps can be used as a reference to identify cell-specific transcriptional changes in congenital heart diseases as well as potentially revealing novel therapeutic targets.
| Authors                  | PMID             | Date           | # of Cells and/or Nuclei | Isolation Method                          | Sequencing Technology                              | Target Cell Types                      | Context                                                                 |
|--------------------------|------------------|----------------|--------------------------|-------------------------------------------|--------------------------------------------------|----------------------------------------|----------------------------------------------------------------------------|
| Li et al. [23]           | PMID: 25633351   | January 2015   | 448 cells                | enzymatic digestion, FACS                 | Manual cell lysis/cDNA preparation; targeted sc-qPCR Fluidigm Dynamic Array IFCs [6] | CMs, ECs, CFs, SMCs                     | Comparison of embryo (E10.5)- and mES-derived cardiac progenitor and CM differentiation |
| Kokkinopoulos et al. [24]| PMID: 26469858   | October 2015   | 1088, 12 deep- sequenced | enzymatic digestion                        | Manual cell lysis/cDNA preparation; targeted Taqman® sc-qPCR, Illumina GA IIx | Cells from the heart forming regions in early mouse embryos | Profile the early FHF cardiac progenitors. Early EB to EHF stage (E6-8) |
| DeLaughter et al. [25]   | PMID: 27840107   | November 2016  | ≈1200                    | enzymatic digestion                       | Fluidigm C1 IFCs; Illumina HiSeq2500             | embryonic and post-natal cardiac cells  | Embryonic to postnatal development: E9.5, E11.5, E14.5, E18.5, P0, P3, p21 and comparison with differentiating mESC and hESC |
| Li et al. [26]           | PMID: 27840109   | November 2016  | 2233                     | enzymatic digestion                       | Fluidigm C1 IFCs, Illumina HiSeq2000; targeted sc-qPCR Fluidigm 96x96 Dynamic Array | embryonic cardiac cells                 | Early murine embryo development: E8.5, E9.5, or E10.5 hearts dissected in multiple zones |
| Lescroart et al. [33]    | PMID: 29371425   | January 2018   | 672                      | enzymatic digestion, FACS in lysis buffer | Smart-seq2[8]; Illumina Hi-Seq 2500             | Mouse embryonic derived cardiac progenitor cells (Mesp1⁺) | Early stage of cardiovascular lineage segregation: Mesp1⁺ progenitor from wt and Mesp1 null embryos E6.75-7.25 |
| Xiao et al. [34]         | PMID: 29689192   | April 2018     | 18,166                   | enzymatic digestion                       | Drop-seq [12]; Illumina Nextseq500              | embryonic cardiac cells                 | Role of Hippo signaling in murine embryo development. CTR versus Lats1/2 CKO E13.5 and E14.5 embryos |
| Authors                  | PMID       | Date                | # of Cells and/or Nuclei | Isolation Method                           | Sequencing Technology                                      | Target Cell Types                  | Context                                        |
|-------------------------|------------|---------------------|--------------------------|--------------------------------------------|------------------------------------------------------------|-------------------------------------|-----------------------------------------------|
| Sereti et al. [27]      | PMID: 29467410 | April 2018         | 122                      | mechanical and enzymatic digestion, FACS   | Fluidigm C1 IFCs; Illumina NextSeq 500                      | αMHC⁺  (αMHC-GFP)                  | CM heterogeneity in E9.5, E12.5, and P1 mouse hearts |
| Su et al. [31]          | PMID: 29973725 | July 2018           | 2384                     | mechanical and enzymatic digestion, FACS   | SMART-seq2, Illumina Nextera XT, NextSeq500                 | ApjCreER labeled SV-cells (E12.5–14.5), and Coup-t2OE, SV cells (E14.5) | Coronary artery specification in the SV |
| Chen et al. [38]        | PMID: 30128894 | August 2018        | 152                      | mechanical and enzymatic digestion, mouth pipette | Smart-seq2; Illumina Hi-Seq 4000                          | ventricle from E9.5 heart tube, wt and Mesp1Cre/ x Dgcr8loxP/loxP | Effect of global microRNA KO on cardiac development |
| Hu et al. [53]          | PMID: 30254108 | September 2018     | ≈20,000                  | mechanical nuclei isolation                | sNucDrop-seq [13]                                          | early post-natal cardiac cell nuclei | Postnatal heart development in WT (p6, p10) and ERRα/γ knockout mice (p10, pediatric mitochondrial cardiomyopathy) |
| Jia et al. [28]         | PMID: 30451828 | November 2018      | 421                      | enzymatic digestion, FACS                 | Fluidigm C1 IFCs, ICELL8™ Single-Cell System (Wafergen); Illumina NextSeq 500 | cardiac progenitor cells from Nkx2-5-emGFP and Isl1GFP/⁺ embryos | CPs developmentE7.5, E8.5, and E9.5 embryos |
| Cui et al. [47]         | PMID: 30759401 | February 2019      | 4000                     | enzymatic digestion, mouth-picking, FACS   | Manual cell picking-lysis; STRT-seq [60]; paired-end sequencing Illumina 4000 | human cardiac fetal cells (6, 7, 13, 17 wks); ECs 22 wks, VCs 17 wks | Spatial/temporal analysis of human cardiac development Comparison with mouse data |
| Cao and Spielmann et al. [11] | PMID: 30787437 | February 2019      | 7089-cardiac muscle lineage (2,058,652 total) | Nuclei from snap frozen embryos, no enzymatic digestion, dispersed in 96 well plates | In plate sci-RNA-seq3; NovaSeq (Illumina) | embryonic cells | Mouse organogenic cell atlas (MOCA) E9.5, E10.5, E11.5, E12.5, E13.5, E14.5 |
Table 1. Cont.

| Authors                  | PMID          | Date       | # of Cells and/or Nuclei | Isolation Method          | Sequencing Technology                                      | Target Cell Types              | Context                                                                 |
|--------------------------|---------------|------------|--------------------------|---------------------------|------------------------------------------------------------|-------------------------------|-------------------------------------------------------------------------|
| Li et al. [29]           | PMID: 31142541| June 2019  | >10,500                  | enzymatic digestion       | Chromium Single Cells 3' v2 (10x Genomics); Fluidigm C1 IFCs, Illumina’s HiSeq 2500 and 4000 | embryonic cardiac cells, Isl1-cre/mTmG embryos | Profiling ventricular chambers of E10.5 heart, by dissection and lineage tracing |
| Hill et al. [35]         | PMID: 31201182| June 2019  | 77,122                   | mechanical and enzymatic digestion | Chromium Single Cells 3' v2 (10x Genomics); Illumina NextSeq 500 | embryonic cardiac cells       | E10.5 and E13.5, control and Pitx2 mutant hearts                         |
| Han et al. [37]          | PMID: 31273086| July 2019  | 3600                     | mechanical and enzymatic digestion, FACS | Chromium Single Cells 3' v2 (10x Genomics); Illumina HiSeq 2500 and HiSeq X TEN | embryonic cardiac cells wt and Hand2os1-null | Effect of the lncRNA Hand2os1- on cardiac development                  |
| Yvanka de Soysa et al.   | PMID: 31341279| July 2019  | 36,654                   | Micro-dissection and enzymatic digestion | Chromium Single Cells 3' v2 (10x Genomics); Illumina NextSeq 500 and HiSeq4000 | embryonic cardiac cells       | Effect of congenital mutation on cardiac development (Hand2-null versus wt, E7.75, E8.25, and E9.25 embryos) |
| Liu et al. [45]          | PMID: 31365875| July 2019  | 55,611                   | enzymatic digestion       | Chromium Single Cells 3' v2 (10x Genomics); Illumina NovaSeq 6000 | embryonic cells from the OFT | Murine OFT development (ps47, ps49, ps51)                               |
| Xiong et al. [30]        | PMID: 31221018| August 2019| 2631                     | Micro-dissection and enzymatic digestion, FACS | Smart-seq2; Illumina HiSeq 4000 | embryonic cardiac cells       | Differentiation trajectory and interlineage communication of cardiac progenitor cells from FHF and SHF (E8.25, 8.75, 9.25 embryos) |
| Goodyer et al. [46]      | PMID: 31284824| August 2019| >22,000                  | Micro-dissection and enzymatic digestion | Chromium Single Cells 3' v2 (10x Genomics); Illumina HiSeq4000 | embryonic cardiac cells       | Cardiac conduction system in the embryo E16.5                           |
| Authors                  | PMID       | Date            | # of Cells and/or Nuclei | Isolation Method                              | Sequencing Technology                  | Target Cell Types                  | Context                                                        |
|-------------------------|------------|-----------------|--------------------------|-----------------------------------------------|----------------------------------------|------------------------------------|----------------------------------------------------------------|
| Asp and Giacomello et al. [51] | PMID: 31835037 | December 2019   | 3717                     | Mechanical and enzymatic digestion, FACS in 384w plates | Chromium Single Cells 3' v2 (10x Genomics); Illumina HiSeq2500 | embryonic cardiac cells | Spatio-temporal transcriptomic of developing human heart at different stages |
| Weinberger et al. [32]  | PMID: 32084358 | February 2020   | at least 5000 cardiac B-cells | mechanical and enzymatic digestion, FACS       | Smart-seq2 and TARGET-seq [61]; Illumina NextSeq500 | fluorescent epicardial reporters (e.g., tbx18:myr-eGFP) | Epicardium heterogeneity in zebrafish cardiac development |
| Holowiecki et al. [36]  | PMID: 32094112 | March 2020      | 5300                     | mechanical and enzymatic digestion, FACS       | Chromium 10x; Illumina HiSeq2500          | nkx2.5:ZsYellow⁺ cells at 28 hpf | pbx4 depletion and OFT development in zebrafish |
| Suryawanshi et al. [57] | PMID: 31589297 | July 2020       | 17,747                   | Langendorf enzymatic perfusion                 | Chromium Single Cells 3' v2 (10x Genomics); Illumina HiSeq2500 | whole human fetal hearts | Congenital heart block (CHB): comparison of healthy versus anti-SSA/Ro-associated CHB foetal hearts (mid-gestation) |
3. Cardiac scRNAseq to Elucidate In Vitro Differentiation and Reprogramming

Single-cell technologies have proven to be invaluable tools for defining cellular heterogeneity and lineage commitment in the context of in vitro cell reprogramming and differentiation (Table 2). At least four published studies have focused on the reprogramming of cardiac fibroblasts to induced CMs (iCM). The first [62] used sc-qPCR to study how to improve a seven-factor protocol to obtain iCMs from human fibroblasts [63]. The authors tested the protocol on hESC-derived hCFs and observed that no additional factor could increase the yield of iCMs; however, HAND2 or microRNA-1 could enhance the CM phenotype, as shown by comparison with hESC-derived CM. Soon after, a separate group adopted the Fluidigm C1 technology to profile 574 neonatal murine fibroblasts three days post-transfection with Mef2c, Gata4, and Tbx5 viruses [64]. The authors identified four main transcriptomic states, which were predicted to form a continuum in the transition to iCMs, and they validated their temporal occurrence with population-based gene expression analysis at six time points post-transfection. By differential gene expression analysis, they observed that the acquisition of CM-specific splicing patterns was a key process in the progressive trans-differentiation; and depletion of the splicing factor Ptbp1 could increase reprogramming efficiency. The same group used a similar approach to profile the gene networks and cellular states underlying the more challenging conversion of human adult fibroblasts to iCM [65]. The authors inferred a trajectory to place cells along an axis of pseudotime, which is a measure of how far a cell has progressed along a biological process, which is predicted based on associations between transcriptomic states and characterized by continuous gradients in gene expression. The pseudotemporal trajectory revealed two branches with cells either differentiating to iCM or going back to fibroblasts, and genes involved in the immune response-associated methylation appeared to be crucial for the transition to iCM. Finally, a recent study combined scRNAseq, ATAC-seq, and ChIP-seq (chromatin immunoprecipitation followed by sequencing) for an in-depth profiling of the effect of Mef2c, Gata4, and Tbx5 expression (individual or combined) in embryonic murine fibroblasts reprogrammed to iCM [66].

The process of hiPSCs differentiation to CM has been initially profiled at multiple time points by two distinct studies [67,68], revealing regulators that can enhance CM differentiation [68], and the conditions that could lead to a prevalence of atrial-like versus ventricular-like CMs [67]. A later study selectively profiled hiPSC-derived CMs at the mid and late stage of differentiation (d12, d40), combining RNAseq data obtained by Fluidigm C1, with electrophysiological measurements obtained in a non-invasive way, by transfecting cells with a construct that allows the visualization of changes in polarization as changes in fluorescence intensity (via ArchLight) [69]. The authors concluded that gene expression was insufficient to predict the electrophysiological state of the CMs, but by differential expression between two stages of differentiation, they identified ion-channel regulators that modulate the hiPSC-CM maturation. In particular, genetic ablation of one of these regulators (FHL1) could lead to ventricular-like APs in the differentiating cells. Finally, a more recent study used the progressive and heterogeneous differentiation of hiPSCs to CMs to compare scRNAseq data obtained by Drop-seq versus DroNc-seq [70]. By analyzing in parallel cells from multiple time points, they observed six distinct populations, five of which were identified by both methods, and both datasets could be used to infer similar pseudo-temporal trajectories.

Further, scRNAseq has been employed to analyze patient-derived hiPSC and dissect the cellular basis of cardiomyopathy associated with Duchenne muscular dystrophy (DMD) [71] and the defective cardiac development in Hypoplastic Left Heart Syndrome (HLHS) [56] and a type of Hypoplastic Right Heart Syndrome (HRHS) [58]. DMD is the most common form of muscular dystrophy, it is caused by mutation in the DMD gene, and it generally leads to dilated cardiomyopathy (DCM). The scRNAseq analysis showed that hiPSC-CM from DMD patients presented a transcriptional dysregulation similar to what was observed in patients’ cardiac biopsies and mouse models of DMD, highlighting their utility as a model for drug testing. HLHS is a complex, multifactorial congenital heart
disease [56]. To study its etiology, the authors first profiled cells isolated from different regions of a human fetal heart at 83 days of gestation and confirmed that the expression of genes previously reported as having de novo mutations associated with HLHS was higher in endothelial/endocardial clusters. Then, they used hiPSC-derived EC from healthy and HLHS patients to dissect the molecular basis of endocardial abnormalities, and with the use of in vitro functional assays, they showed that endocardial defects could lead to impaired EndMT and angiogenesis as well as reduced CM proliferation and maturation. HRHS is associated with pulmonary or tricuspid valve atresia, and it is prevalent in the Asian population. To probe a class of HRHS, Pulmonary Atresia with Intact Ventricular Septum syndrome, the authors profiled hiPSC-CM derived from three patients and three healthy controls, cultured in three different conditions: regular cultures, anisotropic organoids, and cardiac tissue strips [58]. The two bioengineered constructs are devised to measure electrophysiological and contractile responses, respectively, and promote CM maturation. The transcriptomic data on hiPSC-CM at different maturation stages revealed a down-regulation of contractile and maturation genes in the HRHS patients and upregulation of immature transcripts, suggesting intrinsic defects in the CMs that could explain the limited RV growth even after interventions to establish the RV–pulmonary arterial connection.

Single-cell transcriptomics of differentiating ESCs has been used to profile the heterogeneity of Mesp1-induced mesodermal cells [72] and hESC-derived epicardial cells [73], to define the steps leading to CM differentiation [74], and to assess mESC-CM maturation post-in vivo transplantation [75]. The process of differentiation of human ESCs to CMs was analyzed by performing scRNAseq over six different stages using the iCell8 platform. The authors reconstructed pseudotemporal trajectory and identified putative ligand–receptor interactions. They observed a crosstalk between cardiac progenitor cells and endocardial cells at d5, leading to the activation of the transcription factor ETS1. By ChIP-Seq and genetic depletion, they showed that ETS1, and therefore the CPs–endocardial cell communication, is important for cardiac lineage commitment [74]. The same group used the ICell8 platform to dissect post-natal CM differentiation [76]. Integrating previously generated scRNAseq on LV cells at p1, p4, p7, and p14 with new data at p56, they identified fibroblasts as a crucial cell type promoting CM differentiation, which they confirmed in vitro with the co-culture of immature CM, isolated from p1 pups, with neonatal or adult fibroblasts. Single-cell analysis has been also used to analyze the reverse process of adult CM reprogramming to mCPs [77] as well as assess the negative effects of nicotine on hESC differentiating into CMs [78], proving to be a valid platform to determine embryonic toxicity; and to establish the best combination of transcription factors to obtain pacemaker-like cells from Nkx2-5+ CPs, which are derived from the transdifferentiation of adult human adipogenic MSCs (hASMSCs) [79].

Similar timeline studies of cellular differentiation are lacking for adult stem/progenitor cells (Table 3). Target sc-qPCR has been used to profile different subpopulations of human cardiac Lin-Sca1+ cells are defined based on the side population phenotype, the expression of CD31 or PDGFRα+, confirming that the Sca1+/PDGFRα+ fraction included the population of clonogenic progenitor cells [80]. An earlier paper analyzed the inverse process in mice, the de-differentiation of CMs to CMs-derived cardiac progenitor cells, using microarrays for single-cell transcriptomic profiling, sc-qPCR for validations, and microarrays for bulk DNA methylation analysis [77]. One of the main applications of stem/progenitor cells is cardiomyoplasty, involving transplantation in the infarcted myocardium to promote regeneration. At least two studies employed single-cell transcriptomic analysis to assess the paracrine function of progenitor cells injected in mice heart subjected to coronary ligation or sham-operated [81,82]. The first study analyzed bone marrow-derived mesenchymal stem cells, 10 days post-injection, the second analyzed hiPSC-derived CMs 4 days post-injection. Both studies combined bulk transcriptomic data on laser capture microdissected samples, with single-cell Taqman® sc-qPCR through Fluidigm Dynamic Arrays, to show the beneficial factors secreted by the cells of interest, post-injection in the infarcted hearts. More recently, three studies have focused on the controversial population of c-kit+ progenitor
cells [83–85]. In all cases, scRNAseq analysis showed that freshly isolated c-kit+ cells are heterogeneous and include cells with mesenchymal or endothelial features; thus, they are unlikely to differentiate in CMs. By lineage tracing and IHC, the first study suggested a minimal differentiation of c-kit+ cells to CM in response to trans aortic constriction (TAC), which was relatively enhanced in response to doxorubicin-induced cardiac injury [83]. The second study revealed that cultured c-kit+ cells lose the heterogeneity and identity markers of freshly isolated cells and include only two subclusters (expressing cell adhesion and metabolism-related genes, respectively), providing a possible explanation for the limited beneficial effect in clinics [84]. The same group highlighted another possible source of discrepancy between preclinical and clinical studies: the presence of tetraploid c-kit+ cells in rodents but not in pigs or humans [85]. Tetraploid cells escape the replicative senescence, and through scRNAseq, the authors observed that 4N cells tended to express endothelial-related genes, while 2N cells appeared more closely related to fibroblasts.

Overall, these studies show the essential role of single-cell transcriptomics in predicting the function and differentiation fate of stem/progenitor cells in normal conditions and in response to stressors such as injury stimuli, drug treatments, and genetic mutations. The identification of intermediate transcriptomic states provides novel insights into reprogramming and differentiation that are often difficult to interrogate by other means.
| Authors                          | PMID                  | Date       | # of Cells and/or Nuclei | Isolation Method                        | Sequencing Technology                                      | Target Cell Types                  | Context                                                                 |
|---------------------------------|-----------------------|------------|--------------------------|----------------------------------------|------------------------------------------------------------|-----------------------------------|------------------------------------------------------------------------|
| Chan et al. [72]                | PMID: 27131741        | April 2016 | 94                       | enzymatic digestion, FACS (live cells) | Fluidigm C1 IFCs; Illumina HiSeq2500                        | Mesp1-induced embryoid bodies     | Heterogeneity of the Mesp1+ mesoderm cells                              |
| Cho et al. [75]                 | PMID: 28076798        | January 2017 | 24                       | mechanical and enzymatic digestion, FACS | custom plate-based; Illumina NextSeq 500                  | mESCs-derived CMs and adult CMs (αMHC-GFP) | Comparison mESC-derived CMs differentiation, in vitro or post-implantation |
| Bektik et al. [62]              | PMID: 28796841        | August 2017 | Does not specify         | enzymatic digestion from culture, FACS  | Fluidigm C1 IFCs; multiplex TaqMan® sc-qPCR                | hESC-derived hCMs, hCFs and hiCMs (αMHC-mCherry+) | hESC-derived fibroblast differentiation hiCM                              |
| Liu et al. [64]                 | PMID: 29072293        | October 2017 | 454                      | enzymatic digestion, FACS              | Fluidigm C1 IFCs; Illumina HiSeq2500                      | cultured CMs and fibroblasts     | fibroblasts to iCM reprogramming                                      |
| Friedman and Nguyen et al. [68] | PMID: 29072293        | October 2018 | 43,168                   | enzymatic digestion from culture        | Chromium Single Cells 3’ v1(10x Genomics); NextSeq 500 (Illumina), Fluidigm C1 IFCs, Illumina’s HiSeq 2000 | hiPSC-derived CMs                 | Multiple time stages of hiPSC differentiation to CM (day 0, 2, 5, 15, 30). Identify HOPX, signal to enhance CM differentiation |
| Churko et al. [67]              | PMID: 30464173        | November 2018 | 10,427                   | enzymatic digestion from culture        | Chromium Single Cells 3’ v2 (10x Genomics); NextSeq 500 (Illumina). | hiPSC-derived CMs                 | hiPSC-cardiac differentiation. Multiple time points (day 0, 5, 14, and day 45) |
| Biendarra-Tiegs et al. [69]     | PMID: 30892143        | April 2019  | 85                       | enzymatic digestion                    | Fluidigm C1 IFCs, Illumina’s HiSeq 2500                   | hiPSC-derived CM (d12-d40 of differentiation) | hiPSC-derived CM maturation (Electrophysiological vs. Transcriptomic Profiling) |
| Zhou et al. [65]                | PMID: 31230860        | June 2019   | 704                      | enzymatic digestion, FACS live cells   | Fluidigm C1 IFCs, Illumina HiSeq 2500                     | hCF-induced CMs                   | Time course of hCF to hiCM reprogramming (d0, d3, d5, d7, d9 post-infection) |
| Stone et al. [66]               | PMID: 31271750        | July 2019   | 29,718                   | enzymatic digestion, FACS              | Chromium Single Cells 3’ v2 (10x Genomics); Illumina HiSeq 4000 | stimulated embryonic CMs and fibroblasts | Time course of mCF to miCM reprogramming (d-1, 0, 1, 7, 14 post-infection) |
| Raghunathan et al. [79]         | PMID: 31678351        | October 2019 | 560                      | enzymatic digestion from culture        | Chromium Single Cells 3’ v2 (10x Genomics); NextSeq 500 (Illumina). | Induced cardiac pacemaker-like cells | Human CPs (derived from hASMSC) differentiation to pacemaker-like cells |
| Authors                  | PMID          | Date            | # of Cells and/or Nuclei | Isolation Method                                                                 | Sequencing Technology                      | Target Cell Types                                                                 | Context                                                                 |
|-------------------------|---------------|-----------------|--------------------------|---------------------------------------------------------------------------------|---------------------------------------------|-----------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Ruan et al. [74]        | PMID: 31722692| November 2019   | 6879                     | enzymatic digestion; image-based selection of live cells                       | Icell8 platform (Takara); Illumina NextSeq500 | embryonic cardiac cells                                                           | Human ESCs to CM differentiation (d0, 2, 5, 9, 14, and 60)              |
| Gambardella et al. [73] | PMID: 31767620| December 2019   | 362                      | enzymatic from culture                                                           | Smart-seq2; Illumina Nextera XT             | hESC-derived epicardial cells                                                     | Characterization of epicardial cell heterogeneity                         |
| Selewa et al. [70]      | PMID: 32001747| January 2020    | ≈50,000                  | enzymatic digestion from culture, mechanical isolation; Nuclei EZ Prep isolation kit (Sigma) | Drop-seq [12], DroNc-seq [13]; Illumina NextSeq500 | hiPSC-derived CM, human cardiac nuclei                                             | ScRNAseq versus snRNAseq on: iPSC to CM differentiation (d0, 1, 3, 7, 15), human heart tissue |
| Kamdar et al. [71]      | PMID: 32164890| March 2020      | 264                      | enzymatic from culture                                                           | does not specify; Illumina MiSeq            | hiPSC-derived CMs                                                                | CMs derived from control and DMD patients (d30-d90)                     |
| He et al. [78]          | PMID: 32276228| April 2020      | 11,772                   | enzymatic digestion from culture                                                | Chromium Single Cells 3’ v2 (10x Genomics); Illumina HiSeq2000 | hESC-derived cardiac cells                                                        | Effect of nicotine on cardiac differentiation from hESCs                |
| Wang et al. [76]        | PMID: 32444791| May 2020        | 2497                     | enzymatic digestion; image-based selection of live cells                       | Icell8 platform (Takara); Illumina NextSeq500 | murine heart LV; CM-fibroblasts co-cultures                                       | Murine postnatal CM maturation: p1, 4, 7, 14, 56 hearts (LV); in vitro imCM with neonatal or adult fibroblasts |
| Miao et al. [56]        | PMID: 32810435| August 2020     | 32,901 human fetal heart cells (35,284 total)                              | Dissection, mechanical and enzymatical digestion, MACS                         | Chromium Single Cells 3’ v2 (10x Genomics); Illumina HiSeq4000                    | Human fetal heart cell, enrichment for CD144+ endo cells; hiPSC-ECs      | Hypoplastic left heart syndrome (HLHS); human fetal heart tissue, hiPSC-derived endocardium |
| Lam et al. [58]         | PMID: 33089525| October 2020    | 25,079                    | Enzymatic digestion                                                           | Chromium Single Cells 3’ v2 (10x Genomics); Illumina HiSeq4000                     | hiPSC-CMs and hiPSC-CMs in anisotropic sheets or cardiac strips                 | Pulmonary Atresia with Intact Ventricular Septum, a form of HRHS         |
### Table 3. Adult Cardiac Progenitor Cells.

| Authors                  | PMID       | Date       | # of Cells and/or Nuclei | Isolation Method                              | Sequencing Technology                                      | Target Cell Types                             | Context                                      |
|--------------------------|------------|------------|--------------------------|------------------------------------------------|------------------------------------------------------------|-----------------------------------------------|----------------------------------------------|
| Noseda et al. [80]       | PMID: 25980517 | May 2015   | 128                      | enzymatic digestion, FACS                      | Manual RNA extraction; targeted Taqman® sc-qPCR            | Adult cardiac progenitor cells                | Cardiac cell lineage commitment              |
| Yao et al. [81]          | PMID: 26043119 | June 2015  | 48                       | enzymatic digestion, FACS                      | Fluidigm Dynamic Array IFCs targeted sc-qPCR [6]          | Transplanted BM-MSC                          | Paracrine function of injected cells. Analysis 10 days post-ligation |
| Ong et al. [82]          | PMID: 26304668 | August 2015| does not specify         | Langendorff enzymatic digestion, FACS         | Fluidigm Dynamic Array IFCs targeted sc-qPCR [6]          | Transplanted hiPSC-CM                         | Paracrine function of injected cells. Analysis 4 days post-ligation |
| Chen et al. [77]         | PMID: 27622691 | September 2016| 6                        | enzymatic digestion                           | Custom microfluidic chip [86]; Targeted sc-qPCR and MG430 2.0 Affimetrix single-cell transcriptome | CMs, CM-derived progenitor cells (mCPCs)    | CM de-differentiation                        |
| Chen et al. [83]         | PMID: 29021323 | October 2017| 405                      | mechanical and enzymatic digestion, MACS      | Fluidigm C1 IFCs; Illumina HiSeq2500                      | Cardiac CD45−c-kit+ cells                    | Profiling the heterogeneity of c-kit+ CPs, from p1 and adult hearts |
| Kim et al. [84]          | PMID: 30104715 | August 2018| 2465 (10x Chromium); 1126 (Smart-seq2) | mechanical and enzymatic digestion, MACS      | 10x 3' v2 (10x Genomics), Smart-seq2; Illumina HiSeq2500, NextSeq500. | c-Kit+ / Lin− CPs freshly isolated and after 5 passages in culture | Comparison of freshly isolated and cultured CPs |
| Broughton et al. [85]    | PMID: 31231694 | June 2019  | 1664                     | mechanical and enzymatic digestion, FACS      | 10x 3' v2 (10x Genomics); Illumina HiSeq 2500             | c-kit+ interstitial non-myocytes             | Ploidy in cardiac c-kit+ interstitial non-CMs |
4. Profiling Injury Models in Regenerative Heart

The main animal models used to study cardiac regeneration are neonatal mice, within the first week of age [87], and more ancient vertebrates such as zebrafish, which can undergo cardiac regeneration throughout life [88,89] (Table 4). The first pioneering study, presenting single-cell transcriptomic analysis in zebrafish, used the Fluidigm C1 platform to profile about 30 genetically labeled epicardial cells. The epicardial layer had been previously reported to have an essential role in zebrafish heart regeneration. This study highlighted the heterogeneity within this population and revealed a new pan-epicardial marker important for regeneration and injury-induced CM proliferation [90]. Later, CMs from regenerating zebrafish hearts were profiled by SORT-Seq, 7 days post-cryoinjury [91]. The authors observed that CMs in the border zone had a different profile than those in the remote area, resembling embryonic cells, and they presented a metabolic switch to glycolysis promoting proliferation, which was induced by the ErbB2 signaling. Recently, a larger scRNAseq analysis of all ventricular cells has been used to understand the role of Runx1 loss of function mutation in zebrafish cardiac regeneration three days post-cryoinjury [92]. Runx1 appeared to affect the function of different cell types, and the mutant showed less myofibroblast-like cells, less collagen deposition, increased fibrinolysis, and overall enhanced regeneration.

As for cardiac regeneration in neonatal mice, the same group has profiled both CM nuclei [93] and interstitial cells [94], in regenerative (p1) and non-regenerative (p7) mice, sham-operated or 1, 3 days post-MI. The first study identified two factors expressed by proliferating CM in regenerative hearts that could confer protection if overexpressed in non-regenerative adult murine hearts. The second provided both transcriptomic and chromatin-accessibility profiles of all the interstitial cells involved in the regeneration, identifying pro-regenerative factors secreted by the epicardium and by macrophages for the reconstruction of cell-specific gene regulatory networks. Transcription factors such as YAP [95] and PITX2 [96] regulate regeneration in neonatal hearts, partly by modulating antioxidant scavengers’ gene expression, thus protecting the injured myocardium from ROS. Neonatal hearts from a CM-specific Pitx2 conditional gene knockout presented persistent large scars two months post-MI and adipose infiltrates derived from non-CM cells [97]. Three weeks post-MI, snRNAseq showed a relative increase in a subset of CMs expressing genes associated with oxidative stress response, confirming elevated oxidative stress in Pitx2-deficient hearts [97]. Finally, a separate group used scRNAseq to specifically profile CD45+CD3+ T cell response in regenerative mice [98,99]. In the first study [98], the authors compared naïve T cells isolated from the spleen with activated T cells isolated from the hearts of p3 mice 7 days post-cryoinjury and reported reduced proliferation and an increase in chemotactic factors that could recruit innate immune cells. In the second study, they used the same injury model to compare activated T cells in regenerative p3 hearts (versus non-regenerative p8 heart) and observed elevated fibrotic CD4+ T cells and more Th1 Th17 cells in p8 hearts [99]. By CD4+ cell depletion, they were able to restore regeneration in juvenile p8 mice, but not in adult mice, suggesting the acquisition of a distinct loss of function with development.

In summary, scRNAseq has been used to identify cell-specific transcriptomic differences in regenerative versus non-regenerative hearts. This analysis has highlighted cellular functions that are specific to cardiac regeneration, which present notable future targets for pharmacologic efforts to improve cardiac repair.
### Table 4. Cardiac Injury in Regenerative Models.

| Authors                  | Date           | # of Cells and/or Nuclei | Isolation Method                  | Sequencing Technology                  | Target Cell Types                      | Context                                           |
|--------------------------|----------------|--------------------------|-----------------------------------|----------------------------------------|----------------------------------------|--------------------------------------------------|
| Cao et al. [90]          | December 2015  | 31                       | enzymatic digestion, FACS         | Fluidigm C1 platform, Illumina HiSeq 2000 | tcf21 – nucEGFP +                       | Zebrafish cardiac regeneration                   |
| Li and Tao et al. [97]   | September 2018 | 7849                     | mechanical nuclei isolation       | Chromium Single Cells 3' v2 (10x Genomics); Illumina Nextseq 500 | adult cardiac nuclei                   | Analysis of Pitx2 conditional-KO with P2-MI 60 days post-sham or injury |
| Li et al. [98]           | June 2019      | 581 CD3+ heart T cells (1850 from spleen) | mechanical and enzymatic digestion, FACS | Chromium Single Cells 3' v2 (10x Genomics); Illumina HiSeq 2500 | CD3+ T-cells                            | Comparison of naïve T-cells (liver) and Treg (heart) d7 after cryoinjury in P3 mice hearts |
| Honkoop and de Bakker et al. [91] | December 2019 | 768                      | mechanical and enzymatic digestion | SORT-seq [9], plate-based            | embryonic, adult zebrafish cardiac cells | Comparison of embryonic (2dpf) and regenerating CMs (7d cryoinjury) |
| Cui et al. [93]          | March 2020     | 21,737                   | mechanical and enzymatic digestion, nuclei isolation, FACS | Chromium Single Cells 3' v2 (10x Genomics); Illumina Nextseq 500 | CM nuclei                               | Neonatal and postnatal regenerative capacity: CM from P1 or P8 mice sham, d1, d3 post-MI |
| Koth et al. [92]         | April 2020     | 15,415                   | mechanical and enzymatic digestion, FACS | Chromium Single Cells 3' v2 (10x Genomics); Illumina HiSeq 400 | adult zebrafish cardiac cells          | Runx1 KO zebrafish cardiac regeneration          |
| Li et al. [99]           | June 2020      | 2431                     | mechanical and enzymatic digestion, FACS | Chromium Single Cells 3' v2 (10x Genomics); Illumina Nextseq 2500 | cardiac and splenic T-cells            | Neonatal cardiac regeneration after apical resection and cryoinfraction |
| Wang et al. [94]         | December 2020  | 17,320                   | mechanical and enzymatic digestion | Chromium Single Cells 3' v2 (10x Genomics); Illumina Nextseq 500 | Interstitial cells                      | Neonatal and postnatal regenerative capacity: interstitial cells from P1 or P8 mice sham, d1, d3 post-MI |
5. Profiling Cardiac Diseases and Injury Models in Non-Regenerative Hearts

One of the classical applications of transcriptomic analysis is the comparison of healthy versus diseased tissues. Single-cell analysis allows for the characterization of transcriptomic changes during disease within individual cells, but also within entire cell populations that comprise a tissue (Table 5).

Initial cardiac scRNAseq studies on disease models were mainly focused on a specific selected/enriched population of cells. The first attempt to profile the CM response in failing hearts was done on a model of pressure-overload induced through TAC, using the Fluidigm C1 platform [100]. Nuclei were isolated from murine hearts, 8 weeks post-TAC, and from biopsies of patients at the late stage of DCM [100]. Despite the limited number of sequenced cells, the depth of paired-end sequencing and the single-cell resolution led to the identification of two long non-coding intragenic RNAs (lincRNA) that could regulate CM cell cycle re-entry and de-differentiation [100]. A comparable number of full CMs was sequenced, after manual isolation and lysis, with the SmartSeq2 protocol, from sham- and TAC-operated mouse hearts at multiple time points post-surgery (3 days, 1, 2, 4, 8 weeks) [101]. This study identified activated pathways and reconstituted the transcriptional trajectory of remodeling CMs, which showed two distinct fates: adaptation and failing. The same group has later combined scRNAseq by SmartSeq2, with sc-qPCR and smFISH, to obtain spatial information on the heterogenous CM response to pressure overload. They observed that the middle myocardium layer was more affected, with re-expression of the fetal gene *Myh7* [102]. More recently, they have used a similar approach to sequence additional CM from sham and TAC-operated mice, and human patients with severe heart failure (HF) [103], and they have showed that the dopamine receptor 1 is the only catecholamine receptor significantly upregulated in failing hearts and contributes to the ventricular arrhythmia observed in chronic HF patients treated with dopamine. A separate group used an image-based platform to isolate, sequence, and compare mono- and pluri-nucleated CM in homeostasis and 8 weeks post-TAC [104], and they concluded that the differences in ploidy do not correspond to significant transcriptional differences in homeostasis, as well as that the heterogeneity observed post-injury is mainly attributable to the non-homogeneous oxygen distribution.

Two studies have used the SORT-Seq method [9] to profile both CM and non-CM after FACS, sorting them in 384-well plates [105,106]. The first study [105] analyzed the infarct area and border zone region, 3 days post-ischemia/reperfusion (I/R), and the correspondent LV area in control mice, reporting *Cfkd* as a new marker of activated fibroblasts. The second study [106] combined scRNAseq of about 2000 cells, and lineage tracing using a Ki67 knock-in reporter mouse, to investigate proliferating cells in different conditions: adult and neonatal hearts, sham, I/R or MI hearts (scar and distal area) 3-, 7-, and 14-days post-ligation. They found no evidence of quiescent cardiac stem cells or proliferating CM in the adult heart, but the result could be affected by the limited number of cells sampled in each condition. They observed that proliferating adult fibroblasts prevent cardiac rupture post-damage and acquire a neonatal phenotype post-injury with the expression of follistatin-like protein 1 (*Flst1*).

Given their large size and fragile structure, the isolation of CMs by FACS could lead to the capture of damaged cells or cell fragments, especially if the cell sorting is only aimed to remove DAPI+ dead cells [107]. As we describe below, manual or automatic cell dispersion combined with imaging selection, as well as single nuclei isolation, are still considered the most reliable ways to isolate CM for sequencing. The first approach allows for the acquisition of morphological and phenotypic information on the sequenced CMs, including the ploidy level; the second can be applied both on live and frozen tissues, and it is advantageous for a high-throughput sampling of a large number of cells.

The image-based system ICell8 CellSelect (Takara) has been used in two studies [108,109] to automatically select single live nucleated CM and non-CM distributed in nanowells. The first study [108] focused on analyzing CM and non-CM from the atria and ventricle of human healthy, failing, and partially recovering heart post-LVAD (left ventricular assist device) treatment,
providing a resource to investigate inter- and intra-compartmental heterogeneity in response to stress. The second study analyzed the progression toward HF, profiling hearts of mice exposed to TAC at different stages (0, 2, 5, 8, 11 weeks), and showed changes in cell-cell communications, subtype switching in fibroblasts, and activation of pro-inflammatory macrophages in the mid-stage of HF progression that can be targeted to preserve cardiac function. Similar transcriptomic changes were observed in human samples of HF and hypertrophy.

Other studies have used snRNAseq to sample CMs, and they either used total snRNAseq or a combination of snRNAseq and scRNAseq to sequence interstitial cells and CMs in pathological contexts. The first study sequenced nuclei from all cardiac cell types isolated from sham or MI murine hearts 5 days post-surgery. The authors used a tri-transgenic mouse line to differentiate between pre-existing, de novo differentiated, and de-differentiated CM. They observed no de novo CM differentiation from interstitial cells, but a small population of differentiated CM appeared to re-enter the cell cycle. The second study analyzed CM nuclei and interstitial cells in a murine model of hypertension, which was induced with 2-weeks AngII infusion, comparing control and treated mice of both sexes. The analysis revealed the transcriptomic signature of activated fibroblasts responsible for the perivascular and interstitial fibrosis as well as gene expression differences based on biological sex evident in almost all cell types, particularly in fibroblasts. A recent study has used published snRNAseq and scRNAseq on mouse CMs post-TAC and human hearts with DCM, with newly generated scRNAseq data of mouse interstitial cells 7 days post-MI, to prioritize possible biomarkers identified through patient plasma proteomic analysis.

Many studies have selectively focused on the non-CM component of the heart using the high throughput 10x Chromium technology. One early study profiled both primary fibroblasts response to TGFß and the interstitial cells of a genetic mouse model of fibrosis (carrying a mutation in phospholamban PLN<sup>R9C/+</sup>), showing that II11 is a primary downstream effector of TGFß in fibroblasts: its overexpression causes heart and kidney fibrosis, the downregulation protects from fibrosis and organ failure. Two additional studies profiled the interstitial cell response to MI in an unbiased fashion. One analyzed the response of the main cell populations, 3- and 7-days post-ligation, using Pdgfra-GFP mice for the lineage tracing of potential progenitor-like cells. The second profiled seven-time points across the three main phases of cardiac repair: homeostasis (d0), inflammatory (d1), proliferative (d3-d5-d7), and maturation (d14-d28) phase, using a reporter mouse to discriminate epicardial from endocardial-derived fibroblasts (Wt1Cre x ZsGreen mice); and they focused on the different fibroblast types/states prevalent in each phase. Additionally, the authors compared genetically diverse inbred mouse strains characterized by different reparative outcomes to highlight the cell composition and transcriptomic features associated with a higher frequency of cardiac rupture in the transition between the inflammatory and proliferative phases. Three studies selectively profiled the fibroblast component of the heart. The first early study used the Fluidigm C1 platform to profile several hundred CD31-CD45 cells isolated from the scar area of inducible reporter mice for Tcf21 or Postn, 7 days post-MI. Based on the expression of known markers, the authors confirmed that Postn-traced cells accounted for most of the myofibroblasts in the scar and were derived from Tcf21<sup>+</sup> fibroblasts. The second study analyzed cells labeled for Pdgfra and Hic1 expression, in homeostasis and 7 days post-MI, and it showed that the quiescence gene Hic1 is expressed by Sca1<sup>+</sup> Pdgfra<sup>+</sup> cells, which act as progenitors for Sca1 fibrogenic cells post-injury and contribute to pathogenesis rather than regeneration, in contrast to the equivalent cells in skeletal muscle. The second study analyzed Col1a1-GFP<sup>+</sup> fibroblasts in homeostasis and 7, 14, and 30 days post-MI. The authors identified Cd200 as a marker of infarct repair fibroblasts and proved that Chlrc1 expression (previously reported as a marker of myofibroblasts/activated fibroblasts) is essential, since the KO increases the frequency of cardiac rupture, which is a finding confirmed by bulk RNAseq on swine hearts and patients with MI and DCM. The response
of endothelial cells to MI was analyzed in detail, combining clonal lineage tracing with an inducible reporter mouse (Pdgfb-iCreER<sup>T2</sup>-R26R-Brainbow2.1) and scRNAseq on sorted ECs, in homeostasis and 7 days post-ligation [120]. As a result, resident ECs appeared to be the main contributors to new vessels through clonal expansion, and Plvap was identified as a marker of activated/proliferating ECs, which is also expressed in human samples, and it is potentially targetable to improve neovascularization. A recent study has shown that the expression of VEGF-B in CM can stimulate neoangiogenesis and limit the cardiac damage post-MI in adult murine heart [121]. Combining lineage tracing and scRNAseq data, the authors observed that the new vessels were mostly derived from sub-endocardial ECs [121].

Leukocytes have been selectively enriched and profiled in response to MI [122] in a model of pressure–volume overload [123] and experimental myocarditis [124]. The comparison of leukocytes from infarcted and non-infarcted murine hearts 4 days post-surgery has contributed to identifying a subset of interferon-inducible macrophages and an IRF3–interferon axis that could be targeted to reduce inflammation and improve cardiac function [122]. More recently, the same authors have analyzed myeloid cells in the serum of human patients 28 h post-NSTEMI and in mice serum and hearts 1, 2, and 4 days post-MI [125]. They have shown that the expression of interferon-stimulated genes (ISG) starts in the bone marrow and in circulating neutrophils and monocytes (controlled by the transcription factors Tet2 and Irf3), while within the heart, Nrf2 negatively regulates ISG expression in resident Ccr2<sup>+</sup> macrophages. They proposed the use of the ISG score, from blood single-cell analysis, as a prognostic tool to stratify patients who may benefit from anti-inflammatory therapies. CD45<sup>+</sup> leukocytes have also been profiled in a model of pressure-overload induced through TAC, where differences between control hearts and early and late stages of remodeling (1–4 weeks post-surgery) revealed dynamic changes in cells from both the innate and adaptive immune system [123]. Similarly, CD45<sup>+</sup> cells were isolated and sequenced from murine hearts exposed to an experimental autoimmune model (EAM) at different stages: acute, subacute, myopathy phases, and controls [124]. The authors observed a prevalence of macrophages in every stage; neutrophils appeared in the early stage, and T cells mostly appeared in the subacute phase. Both pro-inflammatory macrophages and Th17 cells showed an upregulation of Hif1a, which correlated with the extent of inflammation. Hif1a inhibitor could reduce inflammation in all the stages of EAM, and it could be potentially targeted for treatment, as it was expressed at a higher level in patients with acute myocarditis compared to DCM and controls.

The myeloid cell response to MI has been characterized in depth by first profiling sorted resident macrophages and dendritic cells and then sequencing randomly isolated mononuclear cells from adult hearts control at 2, 11, and 28 days post-MI [126]. This study showed that resident embryonic-derived CCR2<sup>+</sup> macrophages have a non-redundant cardioprotective function that cannot be compensated by the highly similar monocyte-derived TIMD4<sup>+</sup>CCR2<sup>+</sup> macrophages post-MI. In human, peripheral CD31<sup>+</sup> monocytes were profiled in patients with HF with reduced ejection fraction (HFrEF) and healthy individuals, revealing profound phenotypic differences, which were validated with different techniques that could be used as a prognostic tool [127].

Recent advances in single-cell technologies are presenting new opportunities to capture increasing amounts and types of information simultaneously from the same cell. For example, cellular indexing of transcriptomes and epitopes by sequencing (CITEseq) quantifies the expression of surface antigen using DNA-barcoded antibodies, while paired full-length sequencing of T or B cell receptors reveals the specific clonotype of adaptive immune cells.

CITEseq has been used to profile the dynamic changes of neutrophils in response to MI. CD11b<sup>+</sup> live cells were sorted from murine hearts and blood 1, 3, and 5 days post-MI. DNA barcoded antibodies for LY6G, CD64, and LY6C were used to distinguish between neutrophils and monocytes/macrophages [128]. Heart-infiltrating neutrophils appeared to acquire a distinct signature (SiglecF<sup>hi</sup>), while circulating ones underwent aging by d3-d5.
Paired single-cell TCR- and 5′ gene expression sequencing has been recently applied to profile the clonotype of CD4⁺ T cells isolated from the heart and spleen of mice 7 days post-MI. Interestingly, the authors observed that Treg cells are mostly recruited to the heart from the circulating pool, and that they acquire unique features in the tissue, proliferate locally by clonal expansion, and contribute to collagen deposition and repair [129].

Overall, single-cell transcriptomic approaches have been widely employed to study cardiovascular disease, either with a targeted approach, to profile cell-specific changes in response to injury or in an unbiased fashion. The latter studies have been used to address a wide variety of questions, including defining organ-wide changes in cell composition and cell–cell communications, as well as pinpointing how these processes may differ based on biological sex or genetic background. Together, these data provide valuable insights on possible therapeutic targets. As the technology becomes more accessible, compositional shifts and transcriptomic patterns associated with disease could also be used for diagnostic and prognostic screenings.
| Authors            | PMID                          | Date       | # of Cells and/or Nuclei | Isolation Method                      | Sequencing Technology                        | Target Cell Types          | Context                                                                 |
|--------------------|-------------------------------|------------|--------------------------|---------------------------------------|---------------------------------------------|-----------------------------|-------------------------------------------------------------------------|
| Kanisicak et al.   | PMID: 27447449                | July 2016  | 185                      | mechanical and enzymatic digestion, FACS | Fluidigm C1 IFCs; Illumina HiSeq2500        | CD31-CD45- cardiac cells    | Tcf21 lineage tracing during adult MI, TAC and/or AngII infusion       |
| See et al.         | PMID: 28790305                | August 2017| 359                      | mechanical nuclei isolation           | Fluidigm C1 IFCs; Illumina HiSeq2500        | Adult human and murine CMs | CM response to heart failure: Human DCM, mouse TAC (8 weeks)           |
| King et al.        | PMID: 29106401                | November 2017| 4215                   | enzymatic digestion                  | InDrop [14]; Illumina HiSeq2500             | leucocytes                  | IFNγ in leucocytes, CTR and d4 post-MI                                  |
| Schafer et al.     | PMID: 29160304                | November 2017| 4548                   | enzymatic digestion                  | Chromium Single Cells 3' v2 (10x Genomics); NextSeq 500 (Illumina) | adult cardiac non-myocyte  | wt versus PtnRPS1A−/− mouse (cardiac fibrosis phenotype). Il11 mediator of fibroblast activation via TGFβ |
| Gladka, M.M. et al. | PMID: 29386203                | January 2018| 932                     | enzymatic digestion, FACS (DAPI, scatter properties) | plate-based, SORT-seq; Illumina NextSeq | adult CMs, endothelial cells, fibroblasts, and macrophages | Uninjured LV versus ischemic area 3d post-IR. Ck4 regulator of fibroblast activation via TGFβ |
| Nomura et al.      | PMID: 30375404                | October 2018| 482                     | Langendorff perfusion, manual pipette | Manual CM lysis, cDNASeq-smart-seq2; Illumina HiSeq 2500 | adult murine CMs            | CMs response to pressure-overload. Sham, 3d and 1, 2, 4, 8 wks post-TAC |
| Kretzschmar and Post et al. | PMID: 30536045                | December 2018| 1939                   | Mechanical and enzymatic digestion,FACS (DAPI, MitoTracker) | CEL-Seq2 and TruSeq library preparation for NextSeq500 | All murine adult ventricular cells | Ki67-RFP mouse model to assay proliferation during murine cardiac injury |
| Dick et al.        | PMID: 30538539                | December 2018| 8283                   | enzymatic digestion, Ig based FACS on beads enriched CD45+ population | Chromium Single Cells 3' v2 (10x Genomics); Illumina HiSeq2500 | adult mononuclear phagocytes (CD45+ CD64lo–hi CD11bhi) | Profiling macrophages post-murine MI (CTR, d2, d11, d28) |
| Satoh et al.       | PMID: 30611794                | January 2019| 219                     | Langendorff perfusion, manual pipette | Manual cell picking, SMART-seq2, HiSeq 2500 System | adult CMs                  | Spatial and temporal CMs response to pressure-overload. (sham, 1, 2, 8 wks post-TAC) |
| Farbehi et al.     | PMID: 30912746                | March 2019  | 30,118                  | enzymatic digestion, FACS            | Chromium Single Cells 3' v2 (10x Genomics); Fluidigm C1 IFCs, Illumina’s HiSeq 2500 | TIP cells and adult cardiac non-myocytes | Murine MI (Sham, d3, d7)                                                 |
Table 5. Cont.

| Authors                  | PMID       | Date       | # of Cells and/or Nuclei | Isolation Method                          | Sequencing Technology                  | Target Cell Types                        | Context                                                                 |
|--------------------------|------------|------------|--------------------------|-------------------------------------------|-----------------------------------------|------------------------------------------|-------------------------------------------------------------------------|
| Zhang et al. [110]       | PMID: 31231540 | June 2019  | 31,542                   | mechanical isolation and lysis from fresh frozen tissue | 10x Chromium Single Cell 5’ kit (10x Genomics); Illumina HiSeq2500 | adult cardiac nuclei | Murine MI (control and d5), tri-transgenic mouse line for CM lineage tracing |
| Li et al. [120]          | PMID: 31162546 | August 2019| ≃28,000                  | enzymatic digestion, FACS                 | Chromium Single Cells 3’ v2 (10x Genomics); Illumina HiSeq4000 | adult cardiac endothelial cells | Murine MI (control and d7), reporter mouse for clonogenic tracing of ECs |
| Yekelchyk et al. [104]  | PMID: 31399804 | August 2019| 1,301                    | Langendorff enzymatic perfusion           | ICell8 platform (Takara); Illumina Nextera XT | adult CMs mono- and multi-nucleated | CM profiling in CTR hearts and 8-weeks post-TAC                           |
| Martini et al. [123]     | PMID: 31661975 | October 2019| 17,853                   | mechanical and enzymatic digestion, FACS  | Chromium Single Cells 3’ v2 (10x Genomics); Illumina NextSeq500 | Adult cardiac leukocytes | Murine pressure-overload model (1- and 4-weeks post-sham, TAC operation) |
| Wang et al. [108]        | PMID: 31915373 | January 2020| 21,422                   | mechanical and enzymatic digestion, image based live cell selection in 384w plates | ICell8 CellSelect (Takara), plate-based lysis and cDNA synthesis SMARTScribe; Illumina NextSeq500 | adult human CMs and non-CMs from LA/ LV, RV | Human heart failure: healthy donors, HF caused by coronary disease, partial recovery (LVAD treatment) |
| Soliman et al. [117]     | PMID: 31978365 | February 2020| 32,313                   | enzymatic digestion, FACS                 | 10x v3’ v2 (10x Genomics), Illumina NextSeq500 | Pdgfra-eGFP/ Hic1+ cells in homeostasis; Pdgfra-eGFP cells post-MI | Cardiac stromal progenitor response to injury (apical area d0, d7, d14, d28 post-MI) |
| Ren et al. [109]         | PMID: 32098504 | February 2020| 11,492                   | mechanical and enzymatic digestion       | ICell8 CellSelect (Takara), MSND Wafergen | Murine and human heart CMs and non-CMs | Murine pressure-overload model (0, 2, 5, 8, 11 weeks); human heart failure |
| Forte et al. [115]       | PMID: 32130914 | March 2020  | 36,847                   | mechanical and enzymatic digestion, FACS live cells [130] | Chromium Single Cells 3’ v2 (10x Genomics); Illumina HiSeq Xten | Adult murine heart non-myocytes | Murine MI (d0, d1, d3, d5, d7, d14, d28), epicardial lineage tracing, and mouse diversity |
| Abplanalp et al. [127]   | PMID: 32531026 | April 2020  | 181,712                  | MACS magnetic sorting                      | Chromium Single Cells 3’ v2 (10x Genomics); Illumina HiSeq4000 | Human circulating monocytes (CD31+) | Effect of heart failure on circulating monocytes: Healthy versus HFpEF patients |
| Hua et al. [124]         | PMID: 32431172 | May 2020    | 34,665                   | mechanical and enzymatic digestion, FACS live cells | Chromium Single Cells 5’ v2 (10x Genomics); Illumina HiSeq4000 | CD45+ immune cells | Experimental autoimmune myocarditis: d0, d14, d21, d60 post-induction in Balb/c mice |
| Authors                        | PMID          | Date       | # of Cells and/or Nuclei | Isolation Method                          | Sequencing Technology                       | Target Cell Types                      | Context                                                                 |
|-------------------------------|---------------|------------|--------------------------|-------------------------------------------|---------------------------------------------|-----------------------------------------|-------------------------------------------------------------------------|
| McLellan et al. [111]         | PMID: 32795101| July 2020  | 29,558                   | perfusion-based enzymatic digestion, FACS  | Chromium Single Cells 3’ v2 (10x Genomics); Illumina HiSeq4000 | adult cardiac non-myocyte cells and CM nuclei | Murine hypertension (Sham, AngII- 2wks post-treatment), male and female comparison |
| Vafadarnnejad et al. [128]    | PMID: 32811295| August 2020| 1334                     | mechanical and enzymatic digestion, FACS (CD11b+ live cells) | CITE-seq, Chromium Single Cells 3’ v2 and v3 (10x Genomics); Illumina NovaSeq6000 | adult cardiac neutrophils | Neutrophils dynamics post-murine MI (d1, d3, d5)                          |
| Yamaguchi et al. [103]        | PMID: 32868781| August 2020| 280 murine CMs and 514 human CMs | Langendorff enzymatic perfusion, manual pipette | Smart-seq2, Illumina HiSeq2500 | Adult murine and human CMs | Interrogation of cardiac dopamine receptor expression during arrhythmia in mice and heart failure in humans |
| Chan et al. [112]             | PMID: 32883678| September 2020| 830 mouse non-myocyte cells (additionally utilized publicly available data [100,101]) | mechanical and perfusion-based enzymatic digestion, FACS | SMART-Seq2, Illumina HiSeq2000 | LV interstitial cells 7 days post-MI, CM nuclei 8 weeks post-TAC | Identify HF biomarkers combining plasma proteomic analysis and scRNAseq |
| Ruiz-Villalba et al. [118]    | PMID: 32972203| September 2020| 36,858                   | mechanical and enzymatic digestion, FACS (Coll1a1-GFP, CD31, CD45) | 10x Genomics 3’ v2; Illumina NextSeq500 | Adult cardiac fibroblasts, endothelial, immune cells | Murine MI (d0, d7, d30), Cthrc1-KO MI (d7), swine MI (d7) |
| Calcagno and Ng et al. [125]  | PMID: 32978242| Sept 2020  | 10,666 mouse hearts (~145,000 total) | mechanical and enzymatic digestion, FACS (DAPI-, Ter119−) | inDrop [14] and 10x Genomics; Illumina HiSeq2500 and HiSeq4000 | Myeloid cells (neutrophils, monocytes, resident macrophages) | IFNγ in leucocytes: human serum 28h post-NSTEMI, mouse heart d1, d2, d4 post-MI |
| Xia and Lu et al. [129]       | PMID: 32985264| September 2020| 20,755 heart T-cells (and 22,741 spleen T-cells) | mechanical and enzymatic digestion, FACS live cells | Chromium Single Cells 3’ v2 and v3 (10x Genomics); Illumina NovaSeq6000 | murine cardiac and splenic CD4+ T cell TCR sequencing | Profiling Treg in the heart, after MI, I/R, cryoinjury |
| Räsänen et al. [121]         | PMID: 33203221| November 2020| does not specify         | mechanical and enzymatic digestion, FACS  | Chromium Single Cells 3’ v3 (10x Genomics); Illumina NovaSeq6000 | cardiac endothelial cells (CD31+ CD45+ Ter119+ CD140a+ DAPI+) | ECs from CTR and —V—VEGF-B transduced adult hearts |
6. Cardiac scRNAseq Cell Atlases

Single-cell transcriptomic analysis deepens our understanding of tissue complexity as well as the dynamic nature of tissue composition and provides a baseline reference for comparison with diseased states. Numerous healthy single-cell atlases have already been developed using the mouse as a model system, although their scope ranges from studies of a specific cell type (e.g., all endothelial cells, from multiple organs) or organ (e.g., all cells present in the heart), to sampling of almost every tissue type within an organism (Table 6).

In 2018, two groups published the first multi-organ compendia of mouse single-cell data, together comprising 500,000 cells from over 50 different organ or tissue types [10,131]. The first, the Mouse Cell Atlas (MCA) [10], simultaneously described Microwell-Seq, which is a novel scRNAseq method utilizing well-based single-cell capture in agarose. The initial MCA contained \( \approx 5000 \) neonatal heart cells which made up <5% of the total dataset. The website developed in conjunction with this paper has subsequently been updated to MCA 2.0 with additional scRNAseq data from fetal to aged (24 months of age) murine cardiac tissue, totaling over 60,000 heart cells (>800,000 total cells), providing a valuable resource to investigate cardiovascular aging (http://bis.zju.edu.cn/MCA/index.html (accessed on 17 February 2021)). In the second study, the Tabula Muris [131], the authors profiled 100,000 cells from 20 murine tissues using two methods: FACS sorting in plates, combined with Smart-seq2 [8] for sequencing of full transcripts; and microfluidic droplet-based cell isolation, for higher throughput 3′-end short-read sequencing. About 4000 cardiac cells were analyzed with the first method and a few hundred were analyzed with the second one (https://tabula-muris.ds.czbiohub.org/ (accessed on 17 February 2021)). Later, the same consortium used a similar approach to profile 23 organs in male and female mice at six age points with the droplet-based system and three time points using the FACS-based method, ranging from 1 to 30 months [132]. This large dataset, with over 350,000 cells, constitutes the Tabula Muris Senis and includes about 18,282 cardiac cells [132,133]. Transcriptomic changes occurring with age have been also specifically analyzed in the heart, using murine [134] and non-human primate [135] models. The first study [134] profiled 12-week-old and 18-month-old C57BL/6J mice, revealing significant changes in fibroblasts, with upregulation of pro-inflammatory, anti-angiogenic, and osteogenic genes. The second [135] utilized scRNAseq to produce a single-cell compendium of cardiovascular aging in cynomolgus monkeys and reported an increase in inflammatory genes both in immune and non-immune cardiac cells.

The broad scope of studies such as MCA and Tabula Muris enable the interrogation of cell identity and tissue-specific aspects of common cell types such as endothelial cells, fibroblasts, and immune cells. An alternative approach to large, multi-organ total cell atlas studies is to narrow the scope of the investigation to a specific cell type. The murine endothelial cell atlas [136,137] consists of more than 32,000 endothelial cells from 11 different mouse tissues. The analysis of this dataset revealed transcriptomic similarity between tissue pairs, and the marker genes of common, tissue-specific, and new EC subtypes (i.e., IFN-responsive and angiogenic ECs). Cardiac endothelial cells appeared transcriptionally similar to skeletal muscle endothelial cells and enriched for genes involved in membrane transport and redox homeostasis. A database of fibroblasts and vascular mural cells has been obtained by sequencing cells isolated from four muscular tissues, including the heart [138,139]. This study revealed lists of genes that can be used to discriminate between these two mesenchyme-derived cell types. Furthermore, the authors observed a higher degree of heterogeneity among fibroblasts from different organs, compared to mural cells, which was mostly ascribable to differences in the ECM genes and that fibroblast subtypes tend to localize in distinct anatomical positions. In the heart, similarly to other studies [10,113,135], they identified two main fibroblast sub-populations. The smaller sub-population, expressing relatively higher levels of Wif1 and Dkk3, was transcriptionally similar to fibroblasts located in the perimysium, which is the connective tissue surrounding a group of myofibers in the skeletal muscle. In the cardiac context, these cells were mostly localized in the valve and atrioventricular space [138], and they seem to resemble
interstitial valve fibroblasts [140] and endocardial derived fibroblasts [115] described in separate studies. B cells have been profiled in adult heart [141] and compared across multiple tissues [142] (heart, liver, lung, and blood) in order to identify specific gene expression patterns. These data revealed that the naïve organ-associated B-cells share features that distinguish them from circulating B cells, but they also display tissue-specific gene expression patterns of unclear function [142]. Transcriptional changes in cardiac B cells during development reflect an unexpected constant dynamic equilibrium within the B cells from primary lymphoid organs, such as the spleen, even in the absence of injuries [142]. Single-cell analyses such as these facilitate the development of cell-specific atlases and provide insight into cellular identity and organ-specific functions of common cell types.

An alternative to cell type-specific scRNAseq (i.e., all B cells) is an organ-specific approach. One of the first cardiac-specific single-cell atlases reported was by Skelly et al. in 2018 [143]. This study identified cell type diversity within cardiac ventricular tissue from C57BL/6J mice, tested intercellular paracrine support between cardiac fibroblasts and macrophages, and described distinct gene expression profiles between male and female cardiac cells [143]. The study was limited by the cell isolation method, which required an artificial down-sampling of the endothelial cells and excluded CMs and atria. A later series of studies [144–146] used snRNAseq to unbiasedly profile the heart of outbred Fzt:DU mice [144] and compare it to inbred C57BL/6NRj mice heart [145] as well as to previously published Tabula Muris cardiac data [146]. The authors noted significant differences in cardiac tissue composition between strains, namely twice as many total endothelial cells in Fzt:DU cardiac tissue [145], a cluster of “endothelial like CMs” [144], and a small population of possibly cycling CMs [146]; these interesting observations are still awaiting validation and further interrogation.

Additional cardiac scRNAseq studies further narrow the scope to focus on the analysis of organ sub-structures [147] or specific cell-types during uncharacterized processes such as CMs in culture [148,149]. A detailed analysis of the murine sinus node has been recently obtained by combining quantitative proteomic data of nodal and non-nodal atrial tissue, with snRNAseq transcriptomic data [147]. Using this approach, the authors identified enriched ion channel proteins and assigned them to specific cell types, thus shedding light on the molecular basis of the pacemaking activity in nodal CMs. Single-cell analysis of CMs is also useful to study CM behavior in culture. One study profiled the transcriptomic response of primary rat cardiac cells adapting to a 3D culture environment [148], while another analyzed the effect of the structural geometry present during culture on primary neonatal rat CMs gene expression [149]. The authors noted that CMs that were forced to adopt a square shape (as opposed to the endogenous rectangle shape) upregulated markers of cell death and downregulated essential cardiac signaling pathways such as oxidative phosphorylation. Regardless of scope, single-cell atlases are a valuable resource for murine cardiac biology.

Human cardiovascular biology is also aided by the development of healthy single-cell atlases. One of the first reported human heart single-cell atlases included >280,000 nuclei from all four heart chambers from male and female donors [150]. The authors showed significant (and expected) transcriptional differences between atrial and ventricular CMs, as well as a surprising amount of variation in non-myocyte gene expression based on anatomical location such as a unique atrial fibroblast subtype. Additionally, the authors integrated snRNAseq data with Genome-Wide Association Studies (GWAS) cardiometabolic traits and the Druggable Genome [151] to link disease-relevant SNPs and druggable gene targets to specific cell types [152]. The data can be explored from the Broad Institute Single Cell Portal [153]. A more recent human cardiac single-cell atlas comprised nearly 500,000 cells and nuclei from the four chamber walls, as well as the septum and apex [154]. The authors carefully defined the heterogeneous composition of all the main cardiac cell populations and validated the spatial distribution of selected clusters by smFISH. Additionally, they identified the cell types enriched for genes associated with cardiovascular phenotypes and SARS-CoV-2 infection (atrial fibrillation, PR interval, QRS duration, coronary artery
disease, and hypertension diseases) from 12 GWAS studies [155]. The data can be explored at [156].

In summary, scRNAseq of the uninjured heart is a useful tool to better define cardiac cellular identity and tissue composition. Single-cell transcriptomic analysis enables the generation of high-resolution cell atlases to delineate a map of the heart, providing insights into the composition of a healthy heart so as to detect deviations into pathogenesis.
### Table 6. Adult heart single-cell atlases.

| Authors | PMID | Date       | # of Cells and/or Nuclei | Isolation Method                   | Sequencing Technology | Target Cell Types | Context                                                                 |
|---------|------|------------|--------------------------|------------------------------------|-----------------------|--------------------|--------------------------------------------------------------------------|
| Skelly et al. [143] | PMID: 29346760 | January 2018 | 10,519                   | enzymatic digestion, FACS          | Chromium Single Cells 3’ v2 (10x Genomics); Illumina HiSeq 4000 | adult cardiac non-myocyte        | homeostatic murine adult cardiac tissue. Male and female comparison     |
| Han et al. [10] | PMID: 29474909 | February 2018 | 5075 heart cells (Over 400,000 total) | enzymatic digestion | Microwell-Seq; Illumina HiSeq | neonatal cardiac cells | Mouse Cell Atlas                                                        |
| Tabula Muris Consortium [131] | PMID: 30283141 | October 2018 | 4635 heart cells (over 100,000 total) | enzymatic digestion, FACS, manual pipette | GemCode Single-Cell 3’ v2 (10x Genomics) & FACS-based full length transcriptomic; Illumina NovaSeq 6000 | adult cardiac non-myocytes, CM | Homeostatic cell profiling of 20 murine adult organs                   |
| Hulin et al. [140] | PMID: 30796046 | March 2019 | 2840                     | mechanical and enzymatic digestion | Dropseq               | heart valve cells | Aortic valve and mitral valve at P7 and P30                             |
| Linscheid et al. [147] | PMID: 31253831 | June 2019 | 5357                     | Mechanical nuclei isolation | Chromium Single Cells 3’ v3 (10x Genomics); Illumina NovaSeq 6000 | sinus node nuclei | Murine sinus node cell atlas                                           |
| Wang et al. [148] | PMID: 31455969 | August 2019 | 12,865                   | mechanical and enzymatic digestion, FACS | 10x 3’ v2 (10x Genomics); Illumina HiSeq PE150 | 3D-cultured primary cells | Engineered cardiac tissues (derived from rat primary cells)           |
| Vidal et al. [134] | PMID: 31723062 | September 2019 | 27,808                   | Mechanical nuclei isolation | Chromium Single Cells 3’ v3 (10x Genomics); Illumina HiSeq4000 | adult CM and non-myocyte nuclei | Young and aged C57BL/6J mice                                           |
| Haftbaradaran Esfahani et al. [149] | PMID: 31872302 | December 2019 | 435                      | Enzymatic from culture and semi-automatic cell picking [157] | SmartSeq2; Illumina HiSeq 2500 | cultured primary p2 rat CMs | Profiling of CMs with defined morphotypes through custom geometry culture chips [158] |
| Adamo et al. [141] | PMID: 31945014 | January 2020 | 5571                     | mechanical and enzymatic digestion, FACS | Chromium Single Cells 3’ v3 and 5’ V(D)J enriched library (10x Genomics); Illumina NovaSeq6000 | CD45^-Aqua-CD19^+ B-cells | B-cells from the heart and blood of 10-week-old C57BL/6J mice        |
| Wolfen et al. [144] | PMID: 32013057 | January 2020 | 8635                     | mechanical and enzymatic digestion, Nuclei PURE Prep isolation kit (Sigma) | Chromium Single Cells 3’ v3 (10x Genomics); Illumina NovaSeq6000 | adult CM and non-myocyte nuclei | snRNA/seq whole murine heart (Fzt:DU outbred mice)                     |
| Kalucka et al. [136] | PMID: 32059779 | Feb 2020    | 4612 heart endothelial cells (32,567 total cells) | mechanical and enzymatic digestion, FACS | Chromium Single Cells 3’ v2 (10x Genomics); Illumina HiSeq 4000 | Adult murine heart endothelial cells | Murine endothelial cell atlas from 11 tissues                           |
| Authors                        | PMID       | Date    | # of Cells and/or Nuclei | Isolation Method                                                                 | Sequencing Technology                                                                 | Target Cell Types                      | Context                                                                 |
|-------------------------------|------------|---------|--------------------------|----------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|----------------------------------------|------------------------------------------------------------------------|
| Wolfien, Galow, and Müller et al. [145] | PMID: 32243511 | April 2020 | 3464 nuclei (additionally integrated with previously published data [144]) | mechanical and enzymatic digestion, Nuclei PURE Prep isolation kit (Sigma)        | Chromium Single Cells 3’v3 (10x Genomics); Illumina NovaSeq6000                        | adult CM and non-myocyte nuclei       | snRNAseq whole murine heart (Fzt:DU outbred vs. C57BL/6JR mice)       |
| Tucker and Chaffin et al., [150] | PMID: 32403949 | May 2020  | 287, 269                 | mechanical and enzymatic digestion, nuclei isolation                          | Chromium Single Cells 3’ v2 (10x Genomics)                                            | all human heart cell types             | Healthy human adult cardiac tissue: biopsies from four chambers       |
| Rocha-Resende et al. [142]    | PMID: 32663200 | July 2020 | 1004                    | mechanical and enzymatic digestion, FACS                                        | Chromium 10x 3’ v3 and 5’ (10x Genomics); Illumina NovaSeq 6000                      | CD45+ Aqua–CD19+ B-cells              | B-cells from postnatal (2 wks) and adult hearts (8 wks); comparison with other tissues (10 wks) |
| Tabula Muris Consortium [132] | PMID: 32669714 | July 2020 | 18,282 heart cells over 350,000 total (9,669 cells long-reads; 8,613 short-reads) | enzymatic digestion, FACS, manual pipette                                      | GemCode Single-Cell 3’ v2 (10x Genomics) & FACS-based full length transcriptomic; Illumina NovaSeq 6000 | adult cardiac non-myocytes, CM from all four chambers                  | Profiling of 23 murine adult organs over six age stages (1 to 30 months), male and female C57BL/6J.               |
| Muhl et al. [138]             | PMID: 32769974 | August 2020 | 1,279 heart cells (6158 total) | mechanical and enzymatic digestion, FACS                                      | Smart-Seq2; Illumina HiSeq 3000                                                      | PDGFRα+, PDGFRβ+, CD31+               | Comparison of fibroblasts and mural cells from four different organs |
| Ma et al. [135]               | PMID: 32913304 | September 2020 | 42,053 (109,609 additional lung nuclei) | mechanical nuclei isolation and FACS                                          | Chromium Single Cells 3’ v3 (10x Genomics); Illumina NovaSeq6000                    | young and aged primate cardiac nuclei | Lung and heart from young and aged cynomolgus monkeys                   |
| Litviňuková et al. [154]     | PMID: 32971526 | September 2020 | 487,106                 | mechanical and enzymatic digestion, FACS, nuclei isolation                   | Chromium Single Cells 3’ v2 or v3 (10x Genomics); Illumina NextSeq 500 or HiSeq 4000 | adult human CM and non-myocyte nuclei (with selected upsampled whole cells) | Healthy human adult cardiac tissue: biopsies from four chambers plus septum and apex |
7. Single-Cell Analysis and Implications for COVID-19

The global pandemic caused by SARS-CoV-2 has significantly impacted the quality of life for billions of people. The cardiovascular implications of the associated coronavirus disease 2019 (COVID-19) are threefold, as reviewed in [159–162]. First, although viral pneumonia is the most common clinical manifestation of COVID-19, the disease can instigate cardiac injury in diverse forms, including fulminant myocarditis [163–165], MI due to blood-clotting [166], and arrhythmia [167,168]. Additionally, the disease progression appears to be worse in elderly patients with pre-conditions such as diabetes, hypertension, and cardiac diseases [159]. Finally, SARS-CoV-2 utilizes the angiotensin-converting enzyme 2 (ACE2) as the primary entry receptor [169]. ACE2 is an essential component in the renin–angiotensin system (RAS) and is integral in proper blood pressure regulation. It limits the production of the vasoconstrictive AngII by cleaving Angiotensinogen to form Angiotensin 1–7 [170]. Circulating ACE2 is upregulated in different pathological conditions [171], and ACE2 activity seems to be increased in the heart upon treatment with commonly used angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARB) [172]. Given the potential for these anti-hypertensive therapies to increase entry receptors for the virus, their safety was initially questioned [170]. Fortunately, these concerns have been dissipated by clinical trials that showed no significant correlation between the use of these drugs and either the probability of contracting the virus or the severity of the disease [173].

Virus-associated pathologies are the result of complex host–pathogen interactions. scRNAseq could potentially be used to highlight cell-specific responses to viral infection in different tissues. To date, only one preprint manuscript reports the use of scRNAseq on infected human cells from the airway epithelium [174]. Bulk RNAseq has been adopted to profile hiPSC-CMs exposed to SARS-CoV-2 [175]. This study proved that CMs can be directly infected; upregulate genes related to immune response (cytokines such as CXCL2, antiviral genes such as OAS3) and apoptosis; and downregulate genes involved in oxidative phosphorylation, troponins, and the entry receptor ACE2 (similar to previous observations in SARS-CoV-infected myocardium [176]). To our knowledge, no study has yet reported the transcriptomic changes occurring at a single cell level in infected cardiac organoid or tissue biopsies from infected or recovered patients. However, publicly available [177–179] and newly generated [135,154,180–182] single-cell datasets have been interrogated to identify the expression patterns of the entry receptor ACE2 and the proteases necessary for the priming of the viral S-protein (Table 7). These studies showed that hACE2 expression is highest in cardiac pericytes, followed by fibroblast and CMs. The expression of the TMPRSS2 protease is negligible in the heart, but other proteases such as CTSB, CTSL, and FURIN, all of which are expressed at a low level in nearly all cardiac cell types, could act in its place to facilitate virus uptake. The expression of ACE2 is upregulated with aging in non-human primates both in CMs and arterial ECs [135]. It is also upregulated in CMs from patients with heart failure, although there is debate on whether this increased ACE2 expression is restricted to CMs: one study showed a global upregulation of ACE2 in failing hearts at both the RNA and protein level [180], while two others showed that the increase in expression in CMs is compensated by a relative decrease in pericytes and other interstitial cell types [181,182]. In response to ACEi treatment, another study showed a significant upregulation of ACE2 in CMs [181], while a second reported increased expression in all cardiac cell types [182]. Finally, scRNAseq on circulating immune cells in patients with heart failure has shown an increase in monocyte/T cell ratio and dramatic transcriptional changes in the monocyte populations, indicating an enhanced pro-inflammatory state [127].
Table 7. Cardiac Single-cell and COVID-19.

| Authors PMID | Date            | # of Cells and/or Nuclei | Isolation Method                          | Sequencing Technology                                    | Target Cell Types                  | Context                                                                 |
|--------------|-----------------|--------------------------|-------------------------------------------|----------------------------------------------------------|------------------------------------|-------------------------------------------------------------------------|
| Chen et al. [180] PMID: 32227090 | March 2020 | does not specify | enzymatic digestion, nuclei isolation | Chromium Single Cells 3’ v3 (10x Genomics); Illumina Hi-seq Xten | all human heart cell types | hACE2 expression in healthy and failing human hearts |
| Nicin et al. [181] PMID: 32293672 | April 2020 | 57,601 | enzymatic digestion, nuclei isolation | does not specify | all human heart cell types | hACE2 expression in healthy and failing human hearts (1 healthy, 5 aortic stenosis, 2 HFrEF patients) |
| Tucker and Chaffin et al. [182] PMID: 32795091 | June 2020 | 677,785 | mechanical and enzymatic digestion, nuclei isolation | Chromium Single Cells 3’ v3 (10x Genomics) | All human LV cell types | Healthy (n = 11) and failing adult human heart (11 dilated-, 15 hypertrophic- cardiomyopathy) |
Overall, these data provide insight into cardiovascular susceptibility to SARS-CoV-2 infection. They suggest that pericytes may be the first port of entry for the virus in healthy hearts, leading to vascular damage and thrombosis, followed by entry via fibroblasts, which could contribute to worsening diastolic dysfunction. The susceptibility of elderly patients and patients with comorbidities to more severe myocardial injury may be due in part to elevated ACE2 expression and immune system dysregulations.

8. Current Challenges and Future Prospective

As single-cell analyses permeate both public and private research in single-cell analysis, the rapid advancement of novel experimental techniques and algorithmic innovations promises to lead to increasingly precise and efficient tools for genetics and cell biology. Innovation in scRNAseq technology is occurring at an unprecedented rate; multiple scRNAseq methods have already been developed [22], and the number of cells analyzed in cardiac scRNAseq studies is increasing exponentially (Figure 1B). The development of antibody-based cell hashing is enabling multiplexing by indexing samples, which can drive down costs and allow for more complex experimental designs [183]. As single-cell technology advances, it is poised to both become a more accessible tool for researchers and to provide highly resolved portraits of cardiac tissue functional state at homeostasis or in pathological contexts.

A limitation of high-throughput scRNAseq methods is that the data are sparse, with a large fraction of the gene counts equal to zero. Zero counts in scRNAseq can result from technical factors (mRNA is not captured) or can be biological in origin (the gene is not expressed in a particular cell) [184,185]. Several new approaches hold promise for partially ameliorating these limitations. The simultaneous detection of RNA and protein at the single-cell level [17,18] could reveal the presence of stable proteins with few or no cognate transcripts currently present in the cell. This method is particularly useful for precisely defining several immune cell subtypes that are traditionally classified using the expression of surface antigens. Quantitative readouts of chromatin accessibility using snATAC-seq can further support single-cell transcriptomic data [28,64,91]. To maximize the transcriptomic coverage for differential gene expression analysis, a recent study has combined cell-type information from scRNAseq with bulk RNAseq data from the same tissue using a deconvolution method [94].

Commonly used scRNAseq methods generate libraries that are amenable to high-throughput short-read sequencing by capturing the 3′ or 5′ end of each transcript, combined with a tag including the cellular barcode and a unique molecular identifier [8–15]. Short-read sequencing of end-primed transcripts hampers the detection of transcript isoforms, although some scRNAseq methods provide full-length transcript information [186,187]. Methods are currently being developed to combine short-read and long-read sequencing from barcoded cells to identify cell-type specific isoforms through barcode deconvolution [188].

A great advantage of scRNAseq is the ability to observe cells as they progress along a continuum of development, differentiation, or disease. Nevertheless, typical pseudotemporal trajectories inferred from transcriptomic data lack directionality and may not reflect a real biological hierarchy. One novel algorithmic approach to this problem is RNA velocity [189], which harnesses reads derived from introns to assess transcriptional activity at present versus in the past (e.g., unprocessed compared to processed transcript abundances). Moreover, new experimental methods are allowing investigators to combine scRNAseq with clonal lineage tracing by introducing a DNA barcode [190] or CRISPR–Cas9-induced genetic modifications [191,192] in the parental cell.

A continual challenge in single cell biology is the confounding effect of cell isolation, which may introduce changes in gene expression. To compensate for this problem, transcriptomic tools are currently being optimized to enable the preservation of spatial information during RNA capture. There are already multiple platforms for in situ RNAseq, which can be broadly classified as either targeted or unbiased approaches, as reviewed
in [193]. Targeted methods allow for the detection of a limited number of pre-defined targets at high resolution, while unbiased methods can in principle detect the full transcriptome but with limited resolution. One of the first unbiased methods was Spatial Transcriptomics [50], which has been acquired by 10X Genomics and is presently marketed under the Visium tradename. In this approach, tissue sections are placed on glass slides pre-printed with clusters of barcoded primers. The RNA is captured and retrotranscribed in situ, and the RNA–DNA complexes are collected for amplification and sequencing ex situ. The distance between the clusters of primers limits the resolution to regions, currently about 30µm in size, that may include multiple cells. Currently, this approach has been used in two cardiovascular studies, which have sought to bypass limits on resolution by combining spatial transcriptomic data with scRNAseq through computational deconvolution methods in order to reconstitute detailed spatial transcriptomic maps [51,194]. In this fast-developing field, we consider it likely that imaginative new advances will eventually allow unbiased RNA and protein detection on the same tissue section with increasing resolution and sensitivity.

In light of the deluge of recent advances in single cell analysis, it is becoming essential to develop and optimize methods for the integration of data of different types and from different sources [195]. Single-cell isolation methods may vary dramatically between research groups, and this may introduce confounding variables for single-cell study integration. Recently, a series of guidelines on how to report scRNAseq experiments have been published to ensure reproducibility [196]. The standardization of public scRNAseq data submission will facilitate the future integration of many disparate single-cell datasets, which will be a valuable resource for hypothesis generation in cardiovascular biology.

9. Conclusions

Although relatively new, single-cell transcriptomics has already revealed numerous insights in cardiovascular biology. Single-cell analysis of cardiac development has developed our understanding of established key early cardiac transcription factors (Table 1). Single-cell computational trajectories have provided insight into the lineage commitment and differentiation of CPs in vitro (Tables 2 and 3). Numerous cardiac single-cell atlases have been developed in multiple organisms and in multiple contexts including genetic variation, biological sex, and cardiac injury, all of which will provide useful resources for future work (Table 6). Characterizing injury responses in both regenerative and non-regenerative contexts has revealed previously undescribed intermediate cells as well as mechanisms of paracrine signaling, which contribute to regeneration and scar formation (Tables 4 and 5). Disease-related cell-specific transcriptomic signatures can be used as diagnostic and prognostic tools. Most recently, single-cell analysis has provided useful information regarding the cardiac consequences of SARS-CoV-2 infection as multiple groups characterized the expression of the entry receptor hACE2 among cardiac cells (Table 7).

Taken together, single-cell analysis of the heart has revealed previously underappreciated cellular heterogeneity and the importance of paracrine intercellular communication. This diversity of cardiac cell types (and cell subtypes) acting in concert likely contributes to the homeostatic maintenance of cardiac tissue and is integral in the complex biological processes that govern progenitor cell differentiation, cardiovascular development, disease, and regeneration. The use of single-cell analytics will enable the definition of a healthy cardiac cell system and thereby better equip therapeutic pursuit toward the maintenance of this healthy cell system during physiological stress.

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**Abbreviations**

| Acronym | Definition |
|---------|------------|
| AP      | Action potential |
| CFs     | Cardiac fibroblasts |
| CMs     | Cardiomyocytes |
| CPs     | Cardiac progenitors |
| DCM     | Dilated cardiomyopathy |
| E       | Embryonic day |
| EB      | Early allantoic bud stage |
| ECM     | Extracellular matrix |
| ECs     | Endothelial cells |
| EHF     | Early headfold stage |
| ESCs    | Embryonic stem cells |
| FHF     | First heart field |
| HF      | Heart failure |
| HFrEF   | Heart failure with reduced ejection fraction |
| HLHS    | Hypoplastic left heart syndrome |
| hpf     | Hours post fertilization |
| I/R     | Ischemia/reperfusion |
| iCM     | Induced cardiomyocytes |
| IFC     | Integrated fluidic circuits |
| imCM    | Immature CM (p1 hearts) |
| iPSCs   | Induced pluripotent stem cells |
| KO      | Knock-out |
| LRHS    | Hypoplastic right heart syndrome |
| LV      | Left ventricle |
| MACS    | Magnetic activated cell sorting |
| MI      | Myocardial Infarction |
| MSC     | Mesenchymal stem cells |
| NSTEMI  | Non-ST segment elevation myocardial infarction |
| OPT     | Outflow tract |
| p       | Postnatal day |
| ps      | Pairs of somites |
| RNAseq  | Ribonucleic acid sequencing |
| ROS     | Reactive oxygen species |
| RV      | Right ventricle |
| SHF     | Second heart field |
| SMCs    | Smooth muscle cells |
| SV      | Sinus venosus |
| TAC     | Trans aortic constriction |
| UMI     | Unique molecular identifier |
| wt      | Wild type |

**References**

1. Forte, E.; Furtado, M.B.; Rosenthal, N. The interstitium in cardiac repair: Role of the immune–stromal cell interplay. *Nat. Rev. Cardiol.* 2018, 15, 601–616. [CrossRef] [PubMed]

2. Eberwine, J.; Yeh, H.; Miyashiro, K.; Cao, Y.; Nair, S.; Finnell, R.; Zettel, M.; Coleman, P. Analysis of gene expression in single live neurons. *Proc. Natl. Acad. Sci. USA* 1992, 89, 3010–3014. [CrossRef] [PubMed]
29. Li, G.; Tian, L.; Goodyer, W.; Kort, E.J.; Buikema, J.W.; Xu, A.; Wu, J.C.; Jovinge, S.; Wu, S.M. Single cell expression analysis reveals anatomical and cell cycle-dependent transcriptional shifts during heart development. Development 2019, 146, dev173476. [CrossRef]

30. Xiong, H.; Luo, Y.; Yue, Y.; Zhang, J.; Ai, S.; Li, X.; Wang, X.; Zhang, Y.-L.; Wei, Y.; Li, H.-H.; et al. Single-Cell Transcriptomics Reveals Chemotaxis-Mediated Intraorgan Crosstalk During Cardiogenesis. Circ. Res. 2019, 125, 398–410. [CrossRef]

31. Su, T.; Stanley, G.; Sinha, R.; D’Amato, G.; Das, S.; Rhee, S.; Chang, A.H.; Poduri, A.; Raffrey, B.; Dinh, T.T.; et al. Single-cell analysis of early progenitor cells that build coronary arteries. Nat. Cell Biol. 2018, 559, 356–362. [CrossRef] [PubMed]

32. Weinberger, M.; Simões, F.C.; Patient, R.; Sauka-Spengler, T.; Riley, P.R. Functional Heterogeneity within the Developing Zebrafish Epicardium. Dev. Cell 2020, 52, 574–590.e6. [CrossRef] [PubMed]

33. Lescroart, F.; Wang, X.; Lin, X.; Swedlund, B.; Gargouri, S.; Sánchez-Dáñez, A.; Moignard, V.; Dubois, C.; Paulissen, C.; Kinston, S.; et al. Defining the earliest step of cardiovascular lineage segregation by single-cell RNA-seq. Science 2018, 359, 1177–1181. [CrossRef]

34. Xiao, Y.; Hill, M.C.; Zhang, M.; Martin, T.J.; Morikawa, Y.; Wang, S.; Moise, A.R.; Wythe, J.D.; Martin, J.F. Hippo Signaling Plays an Essential Role in Cell State Transitions during Cardiac Fibroblast Development. Dev. Cell 2018, 45, 153–169. [CrossRef] [PubMed]

35. Hill, M.C.; Kadow, Z.A.; Li, L.; Tran, T.T.; Wythe, J.D.; Martin, J.F. A cellular atlas of Pitx2-dependent cardiac development. Dev. 2019, 146, dev180398. [CrossRef] [PubMed]

36. Holowiecki, A.; Linstrum, K.; Ravisankar, P.; Chetal, K.; Salomonis, N.; Waxman, J.S. Pbx4 limits heart size and fosters arch artery formation by partitioning second heart field progenitors and restricting proliferation. Development 2020, 147, dev185652. [CrossRef]

37. Han, X.; Zhang, J.; Liu, Y.; Fan, X.; Ai, S.; Luo, Y.; Li, X.; Jin, H.; Luo, S.; Zheng, H.; et al. The IncRNA Hand2os1/Uph locus orchestrates heart development through regulation of precise expression of Hand2. Development 2019, 146, dev176198. [CrossRef]

38. Chen, X.; Wang, L.; Huang, R.; Qiu, H.; Wang, P.; Wu, D.; Zhu, Y.; Ming, J.; Wang, Y.; Wang, J.; et al. Dgcr8 deletion in the primitive heart uncovered novel microRNA regulating the balance of cardiac-vascular gene program. Protein Cell 2019, 10, 327–346. [CrossRef]

39. Devine, W.P.; Wythe, J.D.; George, M.; Koshiba-Takeuchi, K.; Bruneau, B.G. Early patterning and specification of cardiac progenitors in gastrulating mesoderm. eLife 2014, 3, e03848. [CrossRef]

40. Galdos, F.X.; Wu, S.M. Single-Cell Delineation of Who’s on First and Second Heart Fields During Development. Circ. Res. 2019, 125, 411–413. [CrossRef]

41. Syeda, F.; Kirchhof, P.; Fabritz, L. PITX2-dependent gene regulation in atrial fibrillation and rhythm control. J. Physiol. 2017, 595, 4019–4026. [CrossRef] [PubMed]

42. Ellinor, P.T.; Ibarra-Soria, X.; McDole, K.; Jayaram, S.A.; Godwin, J.; van den Brand, T.A.H.; Miranda, A.M.; Scialdone, A.; Lunetta, K.; Glazer, N.; Pfeufer, A.; Alonso, A.; Chung, M.; Sinner, M.; De Bakker, P.J.; Lubitz, S.; et al. Common variants in KCNN3 are associated with lone atrial fibrillation. Circ. Res. 2019, 125, dev180398. [CrossRef] [PubMed]

43. Cui, Y.; Zheng, Y.; Liu, X.; Fan, X.; Yong, J.; Hu, Y.; Dong, J.; Li, Q.; Wu, X.; et al. The lncRNA Hand2os1/Uph locus orchestrates heart development through regulation of precise expression of Hand2. Development 2019, 146, dev176198. [CrossRef]

44. Tu, J.; Sladek, R.; Carrier, J.; Bader, J.-A.; Richard, D.; Giguère, V. Reduced Fat Mass in Mice Lacking Orphan Nuclear Receptor Estrogen-Related Receptor α. Mol. Cell. Biol. 2003, 23, 7947–7956. [CrossRef]
55. De Soysa, T.Y.; Ranade, S.S.; Okawa, S.; Ravichandran, S.; Huang, Y.; Salunga, H.T.; Schricker, A.; Del Sol, A.; Gifford, C.A.; Srivastava, D. Single-cell analysis of cardiogenesis reveals basis for organ-level developmental defects. Nat. Cell Biol. 2019, 572, 120–124. [CrossRef] [PubMed]
56. Miao, Y.; Tian, L.; Martin, M.; Paige, S.L.; Galdos, F.X.; Li, J.; Klein, A.; Zhang, H.; Ma, N.; Wei, Y.; et al. Intrinsic Endocardial Defects Contribute to Hypoplastic Left Heart Syndrome. Cell Stem Cell 2020, 27, 574–589.e8. [CrossRef]
57. Suryawanshi, H.; Clancy, R.; Morozov, P.; Halushka, M.K.; Buyon, J.P.; Tuschl, T. Cell atlas of the foetal human heart and implications for autoimmune-mediated congenital heart block. Cardiovasc. Res. 2020, 116, 1446–1457. [CrossRef] [PubMed]
58. Lam, Y.; Keung, W.; Chan, C.; Geng, L.; Wong, N.; Brenière-Letuffle, D.; Li, R.A.; Cheung, Y. Single-Cell Transcriptomics of Engineered Cardiac Tissues From Patient-Specific Induced Pluripotent Stem Cell–Derived Cardiomyocytes Reveals Abnormal Developmental Trajectory and Intrinsic Contractile Defects in Hypoplastic Right Heart Syndrome. J. Am. Heart Assoc. 2020, 9, e016528. [CrossRef] [PubMed]
59. Srivastava, D.; Thomas, T.; Lin, Q.; Kirby, M.L.; Brown, D.D.; Olson, E.N. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat. Genet. 1997, 16, 154–160. [CrossRef]
60. Li, L.; Dong, J.; Yan, L.; Yong, J.; Liu, X.; Hu, Y.; Fan, X.; Wu, X.; Guo, H.; Wang, X.; et al. Single-Cell RNA-Seq Analysis Maps Development of Human Germline Cells and Gonadal Niche Interactions. Cell Stem Cell 2017, 20, 891–892. [CrossRef] [PubMed]
61. Rodríguez-Meira, A.; Buck, G.; Clark, S.-A.; Povinelli, B.J.; Alcolea, V.; Louka, E.; McGowan, S.; Hamblin, A.; Sousos, N.; Barkas, N.; et al. Unravelling Intratumoral Heterogeneity through High-Sensitivity Single Tumor Mutation Analysis and Parallel RNA Sequencing. Mol. Cell 2019, 73, 1292–1305. [CrossRef]
62. Bektik, E.; Dennis, A.; Prasanna, P.; Madabhushi, A.; Fu, J.-D. Single cell qPCR reveals that additional HAND2 and microRNA-1 facilitate the early reprogramming progress of seven-factor-induced human myocytes. PLoS ONE 2017, 12, e0183000. [CrossRef]
63. Fu, J.-D.; Stone, N.R.; Liu, L.; Spencer, C.I.; Qian, L.; Hayashi, Y.; Delgado-Olguín, P.; Ding, S.; Bruneau, B.G.; Srivastava, D. Direct Reprogramming of Human Fibroblasts toward a Cardiomyocyte-like State. Stem Cell Rep. 2013, 1, 235–247. [CrossRef]
64. Liu, Z.; Wang, L.; Welch, J.D.; Ma, H.; Zhou, Y.; Vaseghi, H.R.; Yu, S.; Wall, J.B.; Alimohamadi, S.; Zheng, M.; et al. Single-cell transcriptomics reconstructs late conversion from fibroblast to cardiomyocyte. Nat. Cell Biol. 2017, 551, 100–104. [CrossRef]
65. Zhao, Y.; Liu, Z.; Welch, J.D.; Gao, X.; Wang, L.; Garbutt, T.; Keepers, B.; Ma, H.; Prins, J.F.; Shen, W.; et al. Single-Cell Transcriptomic Analyses of Cell Fate Transitions during Human Cardiac Reprogramming. Cell Stem Cell 2019, 25, 149–164.e9. [CrossRef] [PubMed]
66. Stone, N.R.; Gifford, C.A.; Thomas, R.; Pratt, K.J.B.; Samse-Knapp, K.; Mohamed, T.M.A.; Radzinsky, E.M.; Schricker, A.; Ye, L.; Yu, P.; et al. Context-Specific Transcription Factor Functions Regulate Epigenomic and Transcriptional Dynamics during Cardiac Reprogramming. Cell Stem Cell 2019, 25, 87–102.e9. [CrossRef] [PubMed]
67. Kurko, J.M.; Garg, P.; Treutlein, B.; Venkatasubramanian, M.; Wu, H.; Lee, J.; Wessells, Q.N.; Chen, S.-Y.; Chen, W.-Y.; Chetal, K.; et al. Defining human cardiac transcription factor hierarchies using integrated single-cell heterogeneity analysis. Nat. Commun. 2018, 9, 1–14. [CrossRef] [PubMed]
68. Friedman, C.E.; Nguyen, Q.; Lukowski, S.W.; Helfer, A.; Chiu, H.S.; Miklas, J.; Levy, S.; Suo, S.; Han, J.-D.J.; Osteil, P.; et al. Single-Cell Transcriptomic Analysis of Cardiac Differentiation from Human PSCs Reveals HOPX-Dependent Cardiomyocyte Transcriptomic Analysis. Cell Stem Cell 2018, 23, 586–598.e8. [CrossRef] [PubMed]
69. Biendorra-Tiegs, S.M.; Li, X.; Ye, D.; Brandt, E.B.; Ackerman, M.J.; Nelson, T.J. Single-Cell RNA-sequencing and Optical Electrophysiology of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes Reveal Discordance Between Cardiac Subtype-Associated Gene Expression Patterns and Electrophysiological Phenotypes. Stem Cells Dev. 2019, 28, 659–673. [CrossRef]
70. Selawa, A.; Dohn, R.; Eckart, H.; Lozano, S.; Xie, B.; Gauchat, E.; Elorban, R.; Rhodes, K.; Burnett, J.; Gilad, Y.; et al. Systematic Comparison of High-throughput Single-Cell and Single-Nucleus Transcriptomes during Cardiomyocyte Differentiation. Cell Stem Cell 2020, 10, 1–13. [CrossRef] [PubMed]
71. Kamdar, F.; Das, S.; Gong, W.; Kamdar, A.K.; Meyers, T.A.; Shah, P.; Ervasti, J.M.; Townsend, D.; Kamp, T.J.; Wu, J.C.; et al. Stem Cell–Derived Cardiomyocytes and Beta-Adrenergic Receptor Blockade in Duchenne Muscular Dystrophy Cardiomyopathy. J. Am. Coll. Cardiol. 2020, 75, 1159–1174. [CrossRef] [PubMed]
72. Chan, S.S.-K.; Chan, H.H.W.; Kyba, M. Heterogeneity of Mesp1+ mesoderm revealed by single-cell RNA-seq. Biochem. Biophys. Res. Commun. 2016, 474, 469–475. [CrossRef] [PubMed]
73. Gambardella, L.; McManus, S.A.; Moignard, V.; Sebukhan, D.; Delaune, A.; Andrews, S.; Bernard, W.G.; Morrison, M.A.; Riley, P.R.; Göttgens, B.; et al. BNC1 regulates cell heterogeneity in human pluripotent stem cell–derived epicardium. Development 2019, 146, dev17441. [CrossRef]
74. Ruan, H.; Liao, Y.; Ren, Z.; Mao, L.; Yao, E.; Yu, P.; Ye, Y.; Zhang, Z.; Li, S.; Xu, H.; et al. Single-cell reconstruction of differentiation trajectory reveals a critical role of ETS1 in human cardiac lineage commitment. BMC Biol. 2019, 17, 1–16. [CrossRef] [PubMed]
75. Cho, G.-S.; Nishitani, Y.; Tampakakis, E.; Murphy, S.; Andersen, P.; Ousaka, H.; Chelko, S.; Chakir, K.; Hong, I.; Seo, K.; et al. Neonatal Transplantation Confers Maturation of PSC-Derived Cardiomyocytes Conducive to Modeling Cardiomyopathy. Cell Rep. 2017, 18, 571–582. [CrossRef] [PubMed]
76. Wang, Y.; Yao, F.; Wang, L.; Li, Z.; Ren, Z.; Li, D.; Zhang, M.; Han, L.; Wang, S.-Q.; Zhou, B.; et al. Single-cell analysis of murine fibroblasts identifies neonatal to adult switching that regulates cardiomyocyte maturation. Nat. Commun. 2020, 11, 1–18. [CrossRef]
77. Chen, X.; Chakravarty, T.; Zhang, Y.; Li, X.; Zhong, J.F.; Wang, C. Single-cell transcriptome and epigenomic reprogramming of cardiomyocyte-derived cardiac progenitor cells. Sci. Data 2016, 3, 160079. [CrossRef] [PubMed]

78. He, B.; Chen, J.; Tian, M.; Chen, J.; Zhou, C.; Ou, Y.; Wang, S.; Li, X.; Zhuang, J. Adverse effects of nicotine on cardiogenic differentiation from human embryonic stem cells detected by single-cell RNA sequencing. Biochem. Biophys. Res. Commun. 2020, 526, 848–853. [CrossRef]

79. Raghunathan, S.; Islas, J.F.; Mistretta, B.; Iyer, D.; Shi, L.; Gunaratne, P.H.; Ko, G.; Schwartz, R.J.; McConnell, B.K. Conversion of human cardiac progenitor cells into cardiac pacemaker-like cells. J. Mol. Cell. Cardiol. 2020, 138, 12–22. [CrossRef]

80. Noseda, M.; Harada, M.; McSweeney, S.; Leja, T.; Belian, E.; Stuckey, D.J.; Paiva, M.S.A.; Habib, J.; Macaulay, I.; De Smith, A.J.; et al. PDGF-Rα demarcates the cardiogenic clonogenic Sca1+ stem/progenitor cell in adult murine myocardium. Nat. Commun. 2015, 6, 6930. [CrossRef] [PubMed]

81. Yao, Y.; Huang, J.; Geng, Y.; Qian, H.; Wang, F.; Liu, X.; Shang, M.; Nie, S.; Liu, N.; Du, X.; et al. Paracrine Action of Mesenchymal Stem Cells Revealed by Single Cell Gene Profiling in Infarcted Murine Hearts. PLoS ONE 2015, 10, e0129164. [CrossRef]

82. Ong, S.-G.; Huber, B.C.; Lee, W.H.; Kodo, K.; Ebert, A.D.; Ma, Y.; Nguyen, P.K.; Diecke, S.; Chen, W.-Y.; Wu, J.C. Microfluidic Single-Cell Analysis of Transplanted Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes After Acute Myocardial Infarction. Circulation 2015, 132, 762–771. [CrossRef]

83. Chen, Z.; Zhu, W.; Bender, I.; Gong, W.; Kwak, I.-Y.; Yellaami, A.; Hodges, T.J.; Nemoto, N.; Zhang, J.; Garry, D.J.; et al. Pathologic Stimulus Determines Lineage Commitment of Cardiac C-kit+ Cells. Circulation 2017, 136, 2359–2372. [CrossRef]

84. Kim, T.; Echeagaray, O.H.; Wang, B.J.; Casillas, A.; Broughton, K.M.; Kim, B.-H.; Sussman, M.A. In situ transcriptome characteristics are lost following culture adaptation of adult cardiac stem cells. Sci. Rep. 2018, 8, 12060. [CrossRef] [PubMed]

85. Broughton, K.M.; Khieu, T.; Nguyen, N.; Rosa, M.; Mohsin, S.; Quijada, P.; Wang, B.J.; Echeagaray, O.H.; Kubi, D.A.; Kim, T.; et al. Cardiac interstitial tetraploid cells can escape replicative senescence in rodents but not large mammals. Commun. Biol. 2019, 2, 205. [CrossRef] [PubMed]

86. Zhong, J.F.; Chen, Y.; Marcus, J.S.; Scherer, A.; Quake, S.R.; Taylor, C.R.; Weiner, I.P. A microfluidic processor for gene expression profiling of single human embryonic stem cells. Lab Chip 2007, 8, 68–74. [CrossRef]

87. Porrello, E.R.; Mahmoud, A.I.; Simpson, J.A.; Zareie, A.; Olson, E.N.; Sadek, H. Transient Regenerative Potential of the Neonatal Mouse Heart. Science 2011, 331, 1078–1080. [CrossRef]

88. Poss, K.D.; Wilson, L.G.; Keating, M.T. Heart Regeneration in Zebrafish. Science 2002, 298, 2188–2190. [CrossRef]

89. Price, E.L.; Vieira, J.M.; Riley, P.R. Model organisms at the heart of regeneration. Dis. Model. Mech. 2019, 12, dmm040691. [CrossRef] [PubMed]

90. Cao, J.; Navis, A.; Cox, B.D.; Dickson, A.L.; Gemberling, M.; Karra, R.; Bagnat, M.; Poss, K.D.; Sadek, H. Transient Regenerative Potential of the Neonatal Mouse Heart. Science 2011, 331, 1078–1080. [CrossRef]

91. Heallen, T.; Morikawa, Y.; Leach, J.; Tao, G.; Willerson, J.T.; Johnson, R.L.; Martin, J.F. Hippo signaling impedes adult heart regeneration. Development 2016, 143, 232–243. [CrossRef]

92. Honkoop, H.; De Bakker, D.E.; Aharonov, A.; Kruse, F.; Shakked, A.; Nguyen, P.D.; De Heus, C.; Garric, L.; Muraro, M.J.; Shoffner, A.; et al. Single-cell analysis uncovers that metabolic reprogramming by ErbB2 signaling is essential for cardiomyocyte proliferation in the regenerating heart. eLife 2019, 8, 8. [CrossRef] [PubMed]

93. Koth, J.; Wang, X.; Killen, A.C.; Stockdale, W.T.; Potts, H.G.; Bonkhofer, F.; Riley, P.R.; Patient, R.K.; Göttgens, B.; et al. Runx1 promotes scar deposition and inhibits myocardial proliferation and survival during zebrafish heart regeneration. Development 2020, 147, dev18569. [CrossRef]

94. Cui, M.; Wang, Z.; Chen, K.; Shah, A.M.; Tan, W.; Duan, L.; Sanchez-Ortiz, E.; Li, H.; Xu, L.; Liu, N.; et al. Dynamic Transcriptional Responses to Injury of Regenerative and Non-regenerative Cardiomyocytes Revealed by Single-Nucleus RNA Sequencing. Dev. Cell 2020, 53, 102–116.e8. [CrossRef]

95. Wang, Z.; Cui, M.; Shah, A.M.; Tan, W.; Liu, N.; Bassel-Duby, R.; Olson, E.N. Cell-Type-Specific Gene Regulatory Networks Underlying Murine Neonatal Heart Regeneration at Single-Cell Resolution. Cell Rep. 2020, 33, 108472. [CrossRef] [PubMed]

96. Heallen, T.; Morikawa, Y.; Leach, J.; Tao, G.; Willerson, J.T.; Johnson, R.L.; Martin, J.F. Hippo signaling impedes adult heart regeneration. Development 2013, 140, 4683–4690. [CrossRef]

97. Tao, G.; Kahr, P.C.; Morikawa, Y.; Zhang, M.; Rahmani, M.; Heallen, T.R.; Li, L.; Sun, Z.; Olson, E.N.; Amendt, Z.S.B.A.; et al. Pitx2 promotes heart repair by activating the antioxidant response after cardiac injury. Nat. Cell Biol. 2016, 534, 119–123. [CrossRef]

98. Li, L.; Tao, G.; Hill, M.C.; Zhang, M.; Morikawa, Y.; Martin, J.F. Pitx2 maintains mitochondrial function during regeneration to prevent myocardial fat deposition. Development 2018, 145, dev166609. [CrossRef] [PubMed]

99. Li, J.; Yang, K.Y.; Tam, R.C.Y.; Chan, V.W.; Chan, W.Y.; Hori, S.; Zhou, B.; Lui, K.O. Regulatory T-cells regulate neonatal heart regeneration by potentiating cardiomyocyte proliferation in a paracrine manner. Theranostics 2019, 9, 4324–4341. [CrossRef]

100. Li, J.; Jiang, C.; Yang, K.Y.; Huang, X.; Han, M.Y.; Li, X.; Chan, V.W.; Chan, K.S.; Liu, D.; Huang, Z.-P.; et al. Specific ablation of CD4+ T-cells promotes heart regeneration in juvenile mice. Theranostics 2020, 10, 8018–8035. [CrossRef] [PubMed]

101. See, K.; Tan, W.L.W.; Lim, E.H.; Tiang, Z.; Lee, L.T.; Li, P.Y.Q.; Luu, T.D.A.; Ackers-Johnson, M.; Foo, R.S. Single cardiomyocyte nuclear transcriptomes reveal a lincRNA-regulated de-differentiation and cell cycle stress-response in vivo. Nat. Commun. 2017, 8, 1–13. [CrossRef] [PubMed]

102. Nomura, S.; Sato, M.; Fujita, T.; Higo, T.; Sumida, T.; Ko, T.; Yamaguchi, T.; Tobita, T.; Naito, A.T.; Ito, M.; et al. Cardiomyocyte gene programs encoding morphological and functional signatures in cardiomyopathy and failure. Nat. Commun. 2018, 9, 1–17. [CrossRef] [PubMed]
124. Hua, X.; Hu, G.; Hu, Q.; Chang, Y.; Hu, Y.; Gao, L.; Chen, X.; Yang, P.-C.; Zhang, Y.; Li, M.; et al. Single-Cell RNA Sequencing to Dissect the Immunological Network of Autoimmune Myocarditis. *Circulation* 2020, 142, 384–400. [CrossRef]

125. Calçagno, D.M.; Ng, R.P.; Toomu, A.; Zhang, C.; Huang, K.; Aguirre, A.D.; Weissleder, R.; Daniels, L.B.; Fu, Z.; King, K.R. The myeloid type I interferon response to myocardial infarction begins in bone marrow and is regulated by Nr4a2-activated macrophages. *Sci. Immunol.* 2020, 5, eaaz974. [CrossRef]

126. Dick, S.A.; Macklin, J.A.; Nejat, S.; Momen, A.; Clemente-Casares, X.; AlThagafi, M.G.; Chen, J.; Kantores, C.; Hosseinzadeh, S.; Aronoff, L.; et al. Self-renewing resident cardiac macrophages limit adverse remodeling following myocardial infarction. *Nat. Immunol.* 2019, 20, 29–39. [CrossRef]

127. Abplanalp, W.T.; John, D.; Cremer, S.; Assmus, B.; Dorsheimer, L.; Hoffmann, J.; Becker-Pergola, G.; A Rieger, M.; Zeiher, A.M.; Vasa-Nicotera, M.; et al. Single-cell RNA-sequencing reveals profound changes in circulating immune cells in patients with heart failure. *Cardiowas. Res.* 2021, 117, 484–494. [CrossRef]

128. Vidal, R.; Wagner, J.U.G.; Braeuning, C.; Fischer, C.; Patrick, R.; Tombor, L.; Muhly-Reinholz, M.; John, D.; Kliem, M.; Conrad, T.; et al. Transcriptional heterogeneity of fibroblasts is a hallmark of the aging heart. *JCI Insight* 2019, 4. [CrossRef]

129. Xia, N.; Lu, Y.; Gu, M.; Li, N.; Liu, M.; Jiao, J.; Zhu, Z.; Li, J.; Li, D.; Tang, T.; et al. A Unique Population of Regulatory T Cells in Heart Potentiates Cardiac Protection From Myocardial Infarction. *Circulation* 2020, 142, 1956–1973. [CrossRef]

130. Forte, E.; Daigle, S.; Rosenthal, N.A. Protocol for Isolation of Cardiac Interstitial Cells from Adult Murine Hearts for Unbiased Single Cell Profiling. *STAR Protoc.* 2020, 1, 100077. [CrossRef] [PubMed]

131. The Tabula Muris Consortium; Overall Coordination; Logistical Coordination; Organ Collection and Processing; Library Preparation and Sequencing; Computational Data Analysis; Cell Type Annotation; Writing Group; Supplemental Text Writing Group; Principal Investigators. Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. *Nature* 2018, 562, 367–372. [CrossRef]

132. The Tabula Muris Consortium A single-cell transcriptomic atlas characterizes ageing tissues in the mouse. *Nat. Cell Biol.* 2020, 583, 590–595. [CrossRef]

133. Available online: https://tabula-muris-senis.ds.czbiohub.org/ (accessed on 17 February 2021).

134. Salomonis, N.; et al. Maturation of heart valve cell populations during postnatal remodeling. *Circulation* 2020, 141, 1–18. [CrossRef]

135. McLeans, G.; Patel, S.; Liu, J.; He, L.; Mocci, G.; Sun, Y.; Gustafsson, S.; Buyandelger, B.; Chivukula, i.e.; et al. Single-cell RNA-sequencing uncovers fibroblast heterogeneity and criteria for fibroblast and mural cell identification and discrimination. *Nat. Commun.* 2020, 11, 1–18. [CrossRef]

136. Available online: https://betsholtzlab.org/Publications/FibroblastMural/database.html (accessed on 17 February 2021).

137. Muhl, L.; Genove, G.; Leptidis, S.; Liu, J.; He, L.; Mocci, G.; Sun, Y.; Gustafsson, S.; Buyandelger, B.; Chivukula, i.e.; et al. Single-cell analysis uncovers fibroblast heterogeneity and criteria for fibroblast and mural cell identification and discrimination. *Nat. Commun.* 2020, 11, 1–18. [CrossRef]

138. Available online: https://www.vibcancer.be/software-tools/EC-atlas (accessed on 17 February 2021).

139. Available online: https://www.vibcancer.be/software-tools/EC-atlas (accessed on 17 February 2021).

140. Xia, N.; Lu, Y.; Gu, M.; Li, N.; Liu, M.; Jiao, J.; Zhu, Z.; Li, J.; Li, D.; Tang, T.; et al. A Unique Population of Regulatory T Cells in Heart Potentiates Cardiac Protection From Myocardial Infarction. *Circulation* 2020, 142, 1956–1973. [CrossRef]

141. Xia, N.; Lu, Y.; Gu, M.; Li, N.; Liu, M.; Jiao, J.; Zhu, Z.; Li, J.; Li, D.; Tang, T.; et al. A Unique Population of Regulatory T Cells in Heart Potentiates Cardiac Protection From Myocardial Infarction. *Circulation* 2020, 142, 1956–1973. [CrossRef]

142. Rocha-Resende, C.; Yang, W.; Li, W.; Kreisel, D.; Adamo, L.; Mann, D.L. Developmental changes in myocardial B cells mirror changes in B cells associated with different organs. *JCI Insight* 2019, 4. [CrossRef]
149. Esfahani, P.H.; Elbeck, Z.; Sagasser, S.; Li, X.; Hossain, M.B.; Talukdar, H.A.; Sandberg, R.; Knöll, R. Cell shape determines gene expression: Cardiomyocyte morphotypic transcriptomes. Basic Res. Cardiol. 2020, 115, 1–15. [CrossRef]

150. Tucker, N.R.; Chaffin, M.; Fleming, S.J.; Hall, A.W.; Parsons, V.A.; Bedi, K.C.; Akkad, A.-D.; Herndon, C.N.; Arduini, A.; Papangeli, I.; et al. Transcriptional and Cellular Diversity of the Human Heart. Circulation 2020, 142, 466–482. [CrossRef] [PubMed]

151. Finan, C.; Gaulton, A.; Kruger, F.A.; Lumbers, R.T.; Shah, T.; Engmann, J.; Galver, L.; Kelley, R.; Karlsson, A.; Santos, R.; et al. The druggable genome and support for target identification and validation in drug development. Sci. Transl. Med. 2017, 9. [CrossRef] [PubMed]

152. Van Der Wijst, M.G.P.; LifeLines Cohort Study; Brugge, H.; De Vries, D.H.; Deelen, P.; Swertz, M.A.; Franke, L.; BIOS Consortium. Single-cell RNA sequencing identifies celltype-specific cis-eQTLs and co-expression QTLs. Nat. Genet. 2018, 50, 493–497. [CrossRef] [PubMed]

153. Available online: https://singlecell.broadinstitute.org/single_cell (accessed on 17 February 2021).

154. Litvinuková, M.; Talavera-López, C.; Maatz, R.; Reichart, D.; Worth, C.L.; Lindberg, E.L.; Kanda, M.; Polanski, K.; Heinig, M.; Lee, M.; et al. Cells of the adult human heart. Nat. Cell Biol. 2020, 588, 466–472. [CrossRef] [PubMed]

155. Watanabe, K.; Mirkov, M.U.; De Leeuw, C.A.; Heuvel, M.P.V.D.; Posthuma, D. Genetic mapping of cell type specificity for complex traits. Nat. Commun. 2019, 10, 1–13. [CrossRef]

156. Available online: https://www.heartcellatlas.org/ (accessed on 17 February 2021).

157. Környei, Z.; Beke, S.; Mihálfy, T.; Jelitai, M.; Kovács, K.; Szabó, Z.; Szabó, B. Cell sorting in a Petri dish controlled by computer vision. Sci. Rep. 2013, 3, 180088. [CrossRef] [PubMed]

158. Esfahani, P.H.; Knöll, R. An Approach to Study Shape-Dependent Transcriptomics at a Single Cell Level. J. Vis. Exp. 2020, 2020, e61577. [CrossRef]

159. Nishiga, M.; Wang, D.W.; Han, Y.; Lewis, D.B.; Wu, J.C. COVID-19 and cardiovascular disease: From basic mechanisms to clinical perspectives. Nat. Rev. Cardiol. 2020, 17, 543–558. [CrossRef]

160. Zheng, Y.-Y.; Ma, Y.-T.; Zhang, J.-Y.; Xie, X. COVID-19 and the cardiovascular system. Nat. Rev. Cardiol. 2020, 17, 259–260. [CrossRef]

161. Madjid, M.; Safavi-Naeini, P.; Solomon, S.D.; Vardeny, O. Potential Effects of Coronaviruses on the Cardiovascular System. JAMA Cardiol. 2020, 5, 831. [CrossRef] [PubMed]

162. Akhmerov, A.; Marbán, E. COVID-19 and the Heart. Circ. Res. 2020, 126, 1443–1455. [CrossRef] [PubMed]

163. Cuomo, V.; Esposito, R.; Santoro, C. Fulminant myocarditis in the time of coronavirus. Eur. Heart J. 2020, 41, 2121. [CrossRef]

164. Hu, H.; Ma, F.; Wei, X.; Fang, Y. Coronavirus fulminant myocarditis treated with glucocorticoid and human immunoglobulin. Eur. Heart J. 2021, 42, 206. [CrossRef]

165. Wei, X.; Fang, Y.; Hu, H. Immune-mediated mechanism in coronavirus fulminant myocarditis. Eur. Heart J. 2020, 41, 1855. [CrossRef] [PubMed]

166. Shi, W.; Lv, J.; Lin, L. Coagulopathy in COVID-19: Focus on vascular thrombotic events. J. Mol. Cell. Cardiol. 2020, 146, 32–40. [CrossRef] [PubMed]

167. Lazzzerini, P.E.; Boutijir, M.; Cepecchi, P.L. COVID-19, Arrhythmic Risk, and Inflammation. Circulation 2020, 142, 7–9. [CrossRef]

168. Chen, C.; Li, H.; Hang, W.; Wang, D.W. Cardiac injuries in coronavirus disease 2019 (COVID-19). J. Mol. Cell. Cardiol. 2020, 145, 25–29. [CrossRef]

169. Hoffmann, M.; Kleine-Weber, H.; Schroeder, S.; Krüger, N.; Herrler, T.; Erichsen, S.; Schiergens, T.S.; Herrler, G.; Wu, N.H.; Nitsche, A.; et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell 2020, 181, 271–280.e8. [CrossRef]

170. Wang, K.; Gheblawi, M.; Oudit, G.Y. Angiotensin Converting Enzyme 2: A Double-Edged Sword. Circulation 2020, 142. [CrossRef]

171. Anguiano, L.; Riera, M.; Pascual, J.; Valdivielso, J.M.; Barrios, C.; Betriu, A.; Mojal, S.; Fernández, E.; Soler, M.J. Circulating angiotensin-converting enzyme 2 activity in patients with chronic kidney disease without previous history of cardiovascular disease. Nephrol. Dial. Transplant. 2015, 30, 1176–1185. [CrossRef] [PubMed]

172. Ferrario, C.M.; Jessup, J.; Chappell, M.C.; Averill, D.B.; Brosnihan, K.; Bridget Tantall, E.; Ann Diz Debra, I.; Gallagher Patricia, E. Effect of Angiotensin-Converting Enzyme Inhibition and Angiotensin II Receptor Blockers on Cardiac Angiotensin-Converting Enzyme 2. Circulation 2005, 111, 2605–2610. [CrossRef]

173. Fernández-Ruiz, J. RAAS inhibitors do not increase the risk of COVID-19. Nat. Rev. Cardiol. 2020, 17, 383. [CrossRef] [PubMed]

174. Ravindra, N.G.; Alfararo, M.M.; Gasque, V.; Wei, J.; Filler, R.B.; Huston, N.C.; Wan, H.; Szegiti-Buck, K.; Wang, B.; Montgomery, R.R.; et al. Single-cell longitudinal analysis of SARS-CoV-2 infection in human bronchial epithelial cells. BioRxiv 2020. [CrossRef]

175. Sharma, A.; Garcia, G.; Wang, Y.; Plummer, J.T.; Morizono, K.; Arumugaswami, V.; Svendsen, C.N. Human iPSC-Derived Cardiomyocytes Are Susceptible to SARS-CoV-2 Infection. Cell Rep. Med. 2020, 1, 100052. [CrossRef]

176. Oudit, G.Y.; Kassiri, Z.; Jiang, C.; Liu, P.P.; Poutanen, S.M.; Penninger, J.M.; Butany, J. SARS-coronavirus modulation of myocardial ACE2 expression and inflammation in patients with SARS. Eur. J. Clin. Investig. 2009, 39, 618–625. [CrossRef] [PubMed]

177. Guo, J.; Wei, X.; Li, Q.; Li, L.; Yang, Z.; Shi, Y.; Qin, Y.; Zhang, X.; Wang, X.; Zhi, X.; et al. Single-cell RNA analysis on ACE2 expression provides insights into SARS-CoV-2 potential entry into the bloodstream and heart injury. J. Cell. Physiol. 2020, 235, 9884–9894. [CrossRef]
178. Liu, H.; Gai, S.; Wang, X.; Zeng, J.; Sun, C.; Zhao, Y.; Zheng, Z. Single-cell analysis of SARS-CoV-2 receptor ACE2 and spike protein priming expression of proteases in the human heart. *Cardiovasc. Res.* 2020, 116, 1733–1741. [CrossRef] [PubMed]

179. Zhou, Y.; Huang, Y.; Song, X.; Guo, X.; Pang, J.; Wang, J.; Zhang, S.; Wang, C. Single-cell transcriptional profile of ACE2 in healthy and failing human hearts. *Sci. China Life Sci.* 2020, 1–4. [CrossRef]

180. Chen, L.; Li, X.; Chen, M.; Feng, Y.; Xiong, C. The ACE2 expression in human heart indicates new potential mechanism of heart injury among patients infected with SARS-CoV-2. *Cardiovasc. Res.* 2020, 116, 1097–1100. [CrossRef]

181. Nicin, L.; Abplanalp, W.T.; Mellentin, H.; Kattih, B.; Tombor, L.; John, D.; Schmitto, J.D.; Heineke, J.; Emrich, F.; Arsalan, M.; et al. Cell type-specific expression of the putative SARS-CoV-2 receptor ACE2 in human hearts. *Eur. Heart J.* 2020, 41, 1804–1806. [CrossRef] [PubMed]

182. Tucker, N.R.; Chaffin, M.; Bedi, K.C., Jr.; Papangeli, I.; Akkad, A.-D.; Arduini, A.; Hayat, S.; Eraslan, G.; Muus, C.; Bhattacharyya, R.P.; et al. Myocyte Specific Upregulation of ACE2 in Cardiovascular Disease: Implications for SARS-CoV-2 Mediated Myocarditis. *Circulation* 2020, 142, 708–710. [CrossRef]

183. Stoeckius, M.; Zheng, S.; Houck-Loomis, B.; Hao, S.; Yeung, B.Z.; Mauck, W.M.; Smibert, P.; Satija, R. Cell Hashing with barcoded antibodies enables multiplexing and doublet detection for single cell genomics. *Genome Biol.* 2018, 19, 1–12. [CrossRef]

184. Haque, A.; Engel, J.; Teichmann, S.A.; Lönnberg, T. A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications. *Genome Med.* 2017, 9, 1–12. [CrossRef] [PubMed]

185. Choi, K.; Chen, Y.; Skelly, D.A.; Churchill, G.A. Bayesian model selection reveals biological origins of zero inflation in single-cell transcriptomics. *Genome Biol.* 2020, 21, 1–16. [CrossRef]

186. Gupta, I.; Collier, P.G.; Haase, B.; Mahfouz, A.; Joglekar, A.; Floyd, T.; Koepmans, F.; Barres, B.; Smit, A.B.; A Sloan, S.; et al. Single-cell isoform RNA sequencing characterizes isoforms in thousands of cerebellar cells. *Nat. Biotechnol.* 2018, 36, 1197–1202. [CrossRef] [PubMed]

187. Hagemann-Jensen, M.; Ziegenhain, C.; Chen, P.; Ramsköld, D.; Hendriks, G.-J.; Larsson, A.J.M.; Faridani, O.R.; Sandberg, R. Single-cell RNA counting at allele and isoform resolution using Smart-seq3. *Nat. Biotechnol.* 2020, 38, 708–714. [CrossRef]

188. Joglekar, A.; Prjibelski, A.; Mahfouz, A.; Collier, P.; Lin, S.; Anna, K.S.; Jordan, M.; Stephen, R.W.; Bettina, H.; Ashley, H.; et al. Cell-type, single-cell, and spatial signatures of brain-region specific splicing in postnatal development. *BioRxiv* 2020. [CrossRef]

189. La Manno, G.; Soldatov, R.; Zeisel, A.; Braun, E.; Hochgener, H.; Petukhov, V.; Lidschreiber, K.; Kastriti, M.E.; Lönnberg, P.; Furlan, A.; et al. RNA velocity of single cells. *Nat. Cell Biol.* 2018, 560, 494–498. [CrossRef] [PubMed]

190. Weinreb, C.; Rodriguez-Fraticelli, A.; Camargo, F.D.; Klein, A.M. Lineage tracing on transcriptional landscapes links state to fate during differentiation. *Science* 2020, 367, eaaw3381. [CrossRef] [PubMed]

191. Spanjaard, B.; Hu, B.; Mitic, N.; Olivares-Chauvet, P.; Janjuha, S.; Ninov, N.; Junker, J.P. Simultaneous lineage tracing and cell-type identification using CRISPR-Cas9-induced genetic scars. *Nat. Biotechnol.* 2018, 36, 469–473. [CrossRef]

192. Chan, M.M.; Smith, Z.D.; Grosswendt, S.; Kretzmer, H.; Norman, T.M.; Adamson, B.; Jost, M.; Quinn, J.J.; Yang, D.; Jones, M.G.; et al. Molecular recording of mammalian embryogenesis. *Nat. Cell Biol.* 2019, 570, 77–82. [CrossRef] [PubMed]

193. Asp, M.; Bergensträhle, J.; Lundeberg, J. Spatially Resolved Transcriptomes—Next Generation Tools for Tissue Exploration. *BioEssays* 2020, 42, e1900221. [CrossRef] [PubMed]

194. Kuppe, C.; Ramirez, F.R.O.; Li, Z.; Hannani, M.T.; Tanevski, J.; Halder, M.; Cheng, M.; Ziegler, S.; Zhang, X.; Preisker, F.; et al. Spatial mul-ti-omic map of human myocardial infarction. *BioRxiv* 2020. [CrossRef]

195. Stuart, T.; Satija, R. Integrative single-cell analysis. *Nat. Rev. Genet.* 2019, 20, 257–272. [CrossRef]

196. Füllgrabe, A.; George, N.; Green, M.; Nejad, P.; Aronow, B.; Fexova, S.K.; Fischer, C.; Freeberg, M.A.; Huerta, L.; Morrison, N.; et al. Guidelines for reporting single-cell RNA-seq experiments. *Nat. Biotechnol.* 2020, 38, 1–3. [CrossRef]