

Complexity of Tracking the Fate of Adult Progenitor Cells

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Biological systems are composed of interdependent structures that act in concert to achieve a large range of responses, which could not be produced by a single unit operating alone. Systems comprise subsystems of progressively smaller size, in which the number of components is gradually reduced until individual elements are considered. The partition of living organisms in organs, parenchymal and interstitial compartments, multicellular clusters, and, lastly, single cells yields the possibility of discriminating phenotypic, molecular, and functional heterogeneity at the interunit level. Recent technological advances with increasing resolution for minute details permit to capture and unmask outliers present in large cell populations. Intercellular heterogeneity cannot be ignored; single cell-based approaches are required for the study of stem cells and the tracking of their progeny.

The biology of hematopoietic stem cells (HSCs) has clarified the information that can be obtained with population-based and individual cell-based methodologies. The relevance of single cell-derived clonal assays for the understanding of the behavior of HSCs was established at the time of their discovery. This early work has set the stage for future research aiming at the recognition of stem cell fate in solid and nonsolid organs, including the heart. It is unfortunate, however, that the simple concept of cardiac stem cells (CSCs) as individual functional entities is ignored, and the pitfalls of population-based studies in small animal models are dismissed often. It is lamentable that these murine studies have been more broadly interpreted to raise notes of caution about the direction of clinical research.¹⁻⁵

The antagonistic position of developmental and molecular biologists is founded on the unconditional trust in negative findings collected in inducible lineage-tracing mice in which the irreversible tagging of the targeted cells is used to define their destiny. In most cases, the labeling system involves Cre-mediated excision of a stop cassette located upstream of a fluorescent reporter gene. Cre recombinase is driven by a cell type-specific promoter, and the activity of the enzyme is

induced by tamoxifen administration. This strategy has shown an unexpected high proclivity for erroneous interpretations when applied to the characterization of adult stem cell fate. Dozens of independent lineage-tracing models of epithelial stem cells in the mammary gland and prostate have failed to define the actual fate of these cells.⁶ Similarly, the documentation of the destiny of neural stem cells in nestin-driven Cre-ERT2 transgenic mice has been problematic. Only a few of these lines display expression of the reporter gene in neurogenic zones of the adult brain, whereas at least 2 nestin-Cre-ERT2 systems show ectopic reporter expression in non-neurogenic areas.⁷

Genetic Fate Mapping and Cardiomyocyte Origin

The process of cardiomyogenesis has been determined in inducible lineage-tracing mice in which Cre-mediated expression of green fluorescent protein is controlled by myocyte-specific¹ or stem cell-specific^{2,5,8} promoters. In the first case, the administration of tamoxifen to MerCreMer-ZEG double heterozygous mice leads to the switch from constitutive β -galactosidase to inducible GFP in cells expressing α -myosin heavy chain (α -MHC).¹ After a pulse of tamoxifen, GFP expression is restricted to a category of α -MHC-positive cells, resulting in cardiac chimerism with coexistence of β -galactosidase-labeled (inactive Cre) and GFP-labeled (active Cre) cells.¹ By this strategy, the myocyte population cannot be evaluated in its totality; the analysis of lineage tracing is restricted to the subset of GFP-positive cells, introducing an artifactual compartmentalization variable.

The reason why a subset of cardiomyocytes does not express the reporter gene is unclear. Whether the presence or absence of GFP is dictated by inherent features of the 2 classes of cells remains to be established. Several hypotheses pointing to the phenotypic and functional heterogeneity of adult cardiomyocytes have been raised but rapidly dismissed.¹ Data in this regard are critical to overcome the intrinsic limitations of MerCreMer-ZEG mice. Fate mapping is a population-based, or worse yet, a subpopulation-based strategy, which offers reliable evidence only when pools of nearly identical cells are considered, a process that does not occur in nature.

The purpose of fate mapping is the analysis of the destiny of the cells located downstream of those expressing Cre-induced GFP. The MerCreMer-ZEG system tests a tautological hypothesis, that is, the origin of labeled myocytes from the pool of GFP-tagged cardiomyocytes. The common presence of GFP in ancestors and descendants makes it impossible to discriminate the 2 cell subsets. On the basis of an unjustified assumption, GFP-positive cardiomyocytes have been considered as the product of self-duplication of pre-existing postmitotic cells, whereas GFP-negative cardiomyocytes have been thought to be derived from progenitor/precursor cells. This

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Nonstandard Abbreviations and Acronyms

CSC	cardiac stem cell
HSC	hematopoietic stem cell
α-MHC	α -myosin heavy chain

theory does not contemplate the possibility that myocyte precursors, positive for c-kit and α -MHC, and transit amplifying cardiomyocytes, positive for α -MHC and cell cycle proteins, are present within the GFP-positive cell pool. Dividing cardiomyocytes are small and mononucleated, a phenotype that is consistent with their origin from stem/progenitor cells. If this probability is excluded, it has to be demonstrated that replicating GFP-positive cardiomyocytes are fully mature cells.

The lack of identifiers of the differentiated state of cardiomyocytes *in situ* makes this task essentially impossible, requiring the isolation of labeled and unlabeled cells and the meticulous analysis of their characteristics, including size, shape, number of nuclei, electromechanical properties, and calcium transient. The acquisition of these structural and functional parameters allows establishing whether GFP-positive and GFP-negative cardiomyocytes share similar traits or whether immature, intermediate, fully developed, and senescent cells are asymmetrically segregated in the 2 populations. The recognition of these properties is critical because high levels of Cre and GFP expression are cardiotoxic and affect the performance and viability of cardiomyocytes. Cre activates the DNA damage response and upregulates p53, resulting in cell apoptosis;⁹ processed peptides of GFP may induce a T-cell immune response with death of labeled cells, questioning the utilization of this fluorescent protein in long-term studies.

It is unusual that 100% Cre-recombination efficiency is achieved in transgenic mice. Unlabeled α -MHC-positive cells constitute blind spots so that valid conclusions on their origin, fate, and function cannot be reached. Similarly, the rate of renewal of labeled cardiomyocytes cannot be established with certainty. Tamoxifen at the doses commonly used to induce Cre activity persists in the organism for weeks after the cessation of treatment. The long half-life of tamoxifen results in prolonged labeling of a significant number of cells,¹⁰ confounding the interpretation of myocyte regeneration when the relative proportion of GFP-positive and GFP-negative cardiomyocytes is used as an exclusive readout.¹

The integration of the MerCreMer transgene² in chromosome 19 results in deletion of a 19 500 bp fragment including the promoter, exon 1, and part of intron 1 of the A1cf gene.¹¹ A1cf is not expressed in homozygous MerCreMer mice, whereas gene haploinsufficiency is present in heterozygous mice. A1cf regulates liver regeneration after injury, a response that is impaired in heterozygous A1cf mice.¹² The levels of A1cf are low in cardiomyocytes, but the deletion of this protein may affect the biological properties of these cells and the degree of myocardial renewal. Short periods of tamoxifen administration mitigate the potential negative effects of A1cf ablation on cardiomyocytes. However, the pharmacokinetics of tamoxifen and its persistence *in vivo* expands significantly and unpredictably the window of treatment.

A glimpse of the crucial limitations inherent to the MerCreMer-ZEG mouse model can be found in the last paragraph of the original publication.¹ However, these considerations, which invalidate the study in its wholeness, fall in areas of visual loss of the supporters of the concept that CSCs have no role in cardiac homeostasis and repair.

c-kit and Cardiomyogenesis

Caution has to be exercised in the evaluation of cardiomyogenesis when the c-kit promoter is selected to target CSCs and track their fate.^{3,5,8} Only positive findings of GFP-expressing myocytes can be interpreted with confidence; labeled myocytes derive from the upstream pool of c-kit-positive, GFP-positive cells in which Cre recombination occurs at the time of tamoxifen administration. However, the magnitude of the process cannot be determined, disqualifying the unequivocal conclusion that c-kit-positive cells minimally contribute cardiomyocytes to the heart.² Regrettably, the attitude of the scientific community toward fate-mapping studies is unreasonably forgiving. Genetic lineage tracing is considered an unbiased protocol, and this false view has led to the unjustified conclusion that a consensus has been reached on myocardial regeneration³ and that the dust has settled on the irrelevant role that c-kit-positive CSCs have in cardiomyocyte formation.⁴

The fundamental premise for the correct identification of the progeny of CSCs is the specificity of the c-kit promoter. The expression of c-kit in the myocardium is not confined to CSCs and their early committed derivatives; mast cells, mesenchymal stem cells, and bone marrow-derived progenitor cells also express c-kit. Activation of the promoter occurs in CSCs and non-CSCs, resulting in the detection of Cre recombinase in cells other than those of interest. Additionally, c-kit-Cre-mediated GFP labeling fails to recapitulate the endogenous expression of c-kit. Kit knock-in mice, generated by insertion of the construct in the start codon of the c-kit locus, show ectopic expression of GFP in mature endothelial cells and terminally differentiated cardiomyocytes.⁵

A relevant aspect, frequently dismissed, concerns the activity of the c-kit promoter in CSCs. The CSC population is composed of subsets with variable degrees of c-kit expression and replicative potential.¹³ Importantly, knock-in strategies result in haploinsufficiency of the targeted gene, decreasing significantly its expression. The percentage of GFP-positive CSCs was not evaluated in the original study, and the recombination efficiency measured in HSCs was used as a reference point.² This cannot be translated to CSCs without caveats; c-kit expression is 50% lower in CSCs than in HSCs. Cre-mediated recombination is a binary event, but this process is influenced by the threshold of recombinase activity. Cre may promote successful DNA cleavage and GFP labeling only in the small pool of CSCs that are brightly positive for c-kit. CSCs with strong expression of c-kit divide rarely,¹⁴ whereas CSCs sustaining cardiomyogenesis are dimly labeled, and Cre recombination may have failed to reach the threshold level. GFP is not expressed in these parental cells, and the cardiomyocytes formed are negative for the reporter protein. In this regard, only the Leydig cells of the testis were GFP positive, whereas differentiating type A (A1–A4), intermediate, and type B spermatogonia,

characterized by lower levels of c-kit were GFP negative.² A decreased size of the testes up to complete atrophy has been found in this model. Areas of altered pigmentation with white belly spots are also present in Kit^{+MCM} mice. These defects are commonly observed in mice with mutated forms of the c-kit receptor and downregulated tyrosine kinase activity. Thus, the absence of GFP does not reflect the lack of a lineage relationship between CSCs and cardiomyocytes.

Another wrong assumption is that tamoxifen does not alter the fate of CSCs. Tamoxifen, at the dose used in lineage tracing, promotes apoptosis of intestinal stem cells, inhibits hepatocyte turnover, and skews the physiological balance of lineage choices in HSCs. For example, death of the targeted stem cells in the intestinal mucosa confers a selective non-physiological growth advantage to a nontargeted stem cell compartment generating an unlabeled progeny.¹⁵ These events may contribute to the discrepancy observed in Kit^{+MCM} and Kit^{CreERT2} mice, which are characterized by the predominant formation of GFP-positive endothelial cells and cardiomyocytes, respectively.^{5,8}

Clonal Readout of Stem Cell Fate Mapping

The phenotypic and functional heterogeneity of CSCs represents an inevitable biological obstacle in population-based lineage-tracing assays. Surface markers that permit the prospective isolation of homogenous CSCs with high level of purity have not been discovered yet. Thus, the reconstruction of the genealogy of CSCs requires the tracking of single stem cells and their progeny over time. This problem can be addressed by using mice carrying multiple Cre-dependent cassettes with reporter genes of different colors. With high probability, individual progenitor cells undergo different Cre-mediated recombination events, leading to distinguishable nuances of colors that enable the tracking of the descendants of a single stem cell. This approach was used to define the contribution of individual c-kit-positive cells to the renewal of the olfactory tissue in Kit^{CreERT2} mice.¹⁶

Heritable genetic lineage tracing can be performed independently from the Cre-lox system to trace the long-term clonal fate of stem cells in vivo. A type of bar coding is based on the semirandom and permanent integration of the viral genome in the genome of the transduced cell. The viral insertion sites are inherited by the progeny of the infected parental cells and occupy an identical position in the genome of mother and daughter cells; they represent clonal tags for the tracking of the destiny of single stem cells. The dynamic pattern of CSC growth and differentiation in vivo was established by injecting a GFP lentivirus in proximity of the atrial and apical niches. Clonal expansion and differentiation of the transduced CSCs was detected in the midregion of the ventricular myocardium during a period of 1 to 5 months, through the recognition of common integration sites in CSCs and mature cells.¹⁷ This was the first study that determined the fate of CSCs in situ.

Conclusions

Historically, the self-renewal and differentiation of CSCs in vivo has been studied by cell transplantation in animal models of myocardial injury. The validity of these experimental

protocols for the analysis of CSC fate has been censured in view of the possibility that the delivered cells acquire a lineage repertoire different from that of CSCs within their physiological environment. The unveiling of a broader or more restricted array of CSC descendants may be dictated by genetic drift and selection pressure with long-term expansion in vitro, a problem that can be overcome by using minimally amplified CSCs. Additionally, disruption of tissue architecture, abnormal cytokine profile, and alterations of the extracellular matrix may override the inherent properties of CSCs. However, CSCs have not been shown to generate undesired noncardiovascular cell lineages or develop tumors in the infarcted heart. The intramyocardial transplantation and the intracoronary delivery of CSCs mimic the human condition and provides the foundations for cell-based clinical trials.¹⁸ Experimental studies do not predict unequivocally the clinical outcome but are indispensable for the development of therapeutic strategies in humans. It is unacceptable that this approach is criticized and genetic fate-mapping studies and the molecular characterization of CSCs is considered obligatory before their implementation in patients. The supporters of this view share the responsibility of delaying progress in the treatment of heart failure, a devastating disease that has reached endemic proportions.

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