Methods 62 (2013) 232–240

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

The nitroreductase system of inducible targeted ablation facilitates cell-specific regenerative studies in zebrafish

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ARTICLE INFO

Article history:
Available online 27 March 2013

Keywords:
Regeneration
Nitroreductase
Zebrafish
Retina
Ablation
Stem cells
Time-lapse

ABSTRACT

At the turn of the 20th century, classical regenerative biology – the study of organismal/tissue/limb regeneration in animals such as crayfish, snails, and planaria – garnered much attention. However, scientific luminaries such as Thomas Hunt Morgan eventually turned to other fields after concluding that inquiries into regenerative mechanisms were largely intractable beyond observational intrigues. The field of regeneration has enjoyed a resurgence in research activity at the turn of the 21st century, in large part due to "the promise" of cultured stem cells regarding reparative therapeutic approaches. Additionally, genomics-based methods that allow sophisticated genetic/molecular manipulations to be carried out in nearly any species have extended organismal regenerative biology well beyond observational limits. Throughout its history, complex paradigms such as limb regeneration – involving multiple tissue/cell types, thus, potentially multiple stem cell subtypes – have predominated the regenerative biology field. Conversely, *cellular* regeneration – the replacement of specific cell types – has been studied from only a few perspectives (predominantly muscle and mechanosensory hair cells). Yet, many of the degenerative diseases that regenerative biology hopes to address involve the loss of individual cell types; thus, a primary emphasis of the embryonic/induced stem cell field is defining culture conditions which promote cell-specific differentiation. Here we will discuss recent methodological approaches that promote the study of cell-specific regeneration. Such paradigms can reveal how the differentiation of specific cell types and regenerative potential of discrete stem cell niches are regulated. In particular, we will focus on how the nitroreductase (NTR) system of inducible targeted cell ablation facilitates: (1) large-scale genetic and chemical screens for identifying factors that regulate regeneration and (2) *in vivo* time-lapse imaging experiments aimed at investigating regenerative processes more directly. Combining powerful screening and imaging technologies with targeted ablation systems can expand our understanding of how individual stem cell niches are regulated. The former approach promotes the development of therapies aimed at enhancing regenerative potentials in humans, the latter facilitates investigation of phenomena that are otherwise difficult to resolve, such as the role of cellular transdifferentiation or the innate immune system in regenerative paradigms.

1. Tissue regeneration in zebrafish

Zebrafish, like many members of the ray-finned fishes (teleosts), have an innate capacity to regenerate tissues (e.g., fins, heart, eye). Combined with amenability to forward genetic screens and reverse genetic techniques (e.g., morpholino 'knock down'), zebrafish are providing key insights into regenerative processes. For instance, analysis of caudal fin regeneration has provided insight into mechanisms regulating blastema formation, tissue outgrowth, and patterning [1]. Similarly, factors regulating blood vessel branching morphogenesis in regenerating fins were identified through a screen for temperature-sensitive mutants [2]. While fin regeneration can be viewed as analogous to limb regeneration, it is the capacity to regenerate heart tissue that firmly set the zebrafish model system on the world stage [3].

In the years since this seminal report, researchers have succeeded in revealing mechanisms regulating heart regeneration. One intriguing finding is that heart muscle regeneration in zebrafish does not require a permanent resident stem cell population. Instead, mature muscle cells dedifferentiate to a stem/progenitor state, proliferate, and their progeny replace damaged cardiac muscle [4]. The British Heart Foundation intends to invest millions to determine if this ability is translational to damaged human heart tissue.

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http://dx.doi.org/10.1016/j.ymeth.2013.03.017

Zebrafish have also been shown to regenerate retinal tissue through a similar mechanism [5]. Following injury, Müller glia cells dedifferentiate to a stem-like state and proliferate to replace lost retinal cells. Importantly, this capacity to repair neural tissue damage is not limited to the eye. Recently, an Australian group demonstrated that zebrafish utilize fibroblast growth factor signaling to repair spinal cord injuries without scarring [6]. The absence of scarring is thought to underlie an enhanced capacity for nervous system repair in zebrafish. The primary emphasis of regenerative studies in the nervous system, however, is on cellular repair (i.e., axonal regeneration) as opposed to whole cell replacement. Despite significance for many degenerative diseases – where significant cell loss often precedes disease detection, thus regeneration stands as the only means to regain lost function – the study of cell-specific regeneration has been far less common than investigations of tissue regeneration and cellular repair.

2. Cell-specific ablation and regeneration in zebrafish

Investigations of mechanosensory hair cell loss and replacement within neuromasts of the lateral line (a peripheral linearly arrayed system of sensory organs) initially determined that the regenerative capacity of zebrafish extends to the level of individual cell types [7]. These studies were facilitated by aminoglycosides (i.e., antibiotics) which are toxic to hair cells, thus providing a simple chemically-induced cell ablation methodology. Moreover, fluorescent dyes that quickly and reproducibly label hair cells (e.g., FM 1–43, To-Pro-3) allow rapid visual assessment of the regenerative process. Such studies have shown that regenerative hair cell progenitors arise from surrounding support cells which purportedly can repopulate lost hair cells through both proliferation-dependent and -independent mechanisms [8].

Genetic screens have succeeded in identifying regeneration-deficient mutants incapable of replacing hair cells [9]. Additionally, chemical modulators that enhance or inhibit this process were recently identified through large-scale compound screens [10], thus providing further molecular insights into which signaling pathways are involved in hair cell regeneration. Similarly, studies in which melanocytes were chemically ablated ultimately revealed that stem cells responsible for melanocyte regeneration are regulated via *kit* receptor tyrosine kinase signaling [11].

Other 'cell toxins' have been used to ablate different cell types (e.g., neuronal subtypes); however, the specificity of such reagents is often problematic. Thus, improved cell ablation techniques have been sought to expand cellular regeneration studies to nearly any desired cell type. Systems promoting the loss or functional compromise of distinct cell types, allow tests of cellular function (e.g., neural subcircuitry 'dissection'), and facilitate investigations of mechanisms permitting cell-specific regeneration.

3. Cell-specific ablation methodologies

Both chemically induced and light induced (e.g., laser ablation) cell ablation paradigms are applied in modern regenerative biology. Their relative strengths and weaknesses facilitate different questions and/or assay endpoints (summarized in Table 1). Typically, chemical ablation provides a less rapid approach with regard to cell death onset, and can also suffer from potential off-site effects (e.g., ill-defined cell 'toxins'). However, the ability to elicit cell loss in large numbers of fish makes this approach applicable to large-scale genetic and/or compound screens. As the entire organism is typically exposed to chemical treatments, utilizing transgenic targeting methodologies in conjunction with chemical ablation can limit undesired "off-site" effects; however, this approach requires additional time to create appropriate toolsets. Light induced ablation techniques offer a rapid onset, but reproducibility also typically requires cell-specific targeting methods (e.g., transgenic reporters, dyes, etc.).

With regard to cell function assays, chemical ablation, by permitting the elimination of defined cells in a large number of fish simultaneously better facilitates studies requiring a large number of samples (e.g., behavioral tests). Conversely, light induced methods are ideal for single cell scales and for high-resolution imaging of cellular responses (e.g., effects on neighboring cells) due to the strict control over the timing of cell loss.

4. Cell-specific ablation systems

4.1. Light/laser ablation

The zebrafish retina provides an ideal system for light ablation as (1), photoreceptor cells are sensitive to intense light and (2) optical transparency can be prolonged via chemical/mutant inhibition of pigment formation into larval and adult stages, respectively. Photoreceptors are especially vulnerable to damage due to their proximity to the retinal pigment epithelium which absorbs the majority of light energy. Thus, prolonged intense light treatments can be used to destroy photoreceptors but spare the inner retina from damage [4].

An alternative method involves Argon laser photoablation. Again, due to their relative sensitivity to light this technique allows selective ablation of photoreceptors [12]. This approach illuminated a role for inner nuclear layer progenitor cells during retinal regeneration. Photoreceptor losses lead to lesion-localized proliferation of microglia, retinal progenitors, and Müller glia – the latter cell type migrated from the inner to the outer nuclear layer, implicating them in the regeneration of cone photoreceptors. Combining reductions in laser intensity and/or exposure times with fluorescent reporter based cell targeting, it is possible to induce highly localized cell loss [13]. This approach can be coupled with confocal imaging to assess immediate effects of cell loss on neighboring cell types and/or resident stem cell niches.

Table 1
Comparison of chemical and light induced ablation methodologies.

Property/methodology	Chemically induced ablation	Light induced ablation
Temporal dynamics	+ (slow or ill defined onset possible)	+++ (rapid)
Specificity	+/+++ (off-site effects possible[a])	+/+++ (facilitated by labeling cell targets)
Sample size	+++ (applicable to multiple fish simultaneously)	+ (labor intensive, limiting sample number)
Reproducibility	+++ (injury proceeds at a constant rate)	++ (variable injury possible)
Behavioral studies	+++ (facilitated by larger sample sizes)	+ (limited by smaller sample sizes)
Imaging studies	++ (dynamics can complicate assay design)	+++ (facilitated by improved dynamics)
Translational impact	+++ (applicable to many degenerative diseases)	+++ (applicable to broad range of injury paradigms)

[a] Typically problematic for toxins as opposed to transgenically targeted methods.