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Toward a blueprint for regeneration

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Summary

Tissue regeneration has been studied for hundreds of years, yet remains one of the less understood topics in developmental biology. The recent Keystone Symposium on Mechanisms of Whole Organ Regeneration brought together biologists, clinicians and bioengineers representing an impressive breadth of model systems and perspectives. Members of the growing regeneration community discussed classic and new ideas on mechanisms of regeneration and how these can be applied to regenerative medicine.

Introduction

Tissue regeneration is one of the earliest fields of reported biological investigation, with scientific luminaries from Spallanzani to Morgan playing key roles in its progression. The recent Keystone Symposium on Mechanisms of Whole Organ Regeneration, organized by Alejandro Sanchez Alvarado (HHMI/Stowers Institute for Medical Research) and held at the mountain resort of Breckenridge, Colorado, was timely, exciting, and above all, inclusive. Although an interest in stem cells courses through the veins of many or all of the participating scientists, this represents just one branch of tissue regeneration biology. How and why tissue regeneration occurs are among the most complex and vexing questions in biology, and there are many steps key to effective regeneration that require research attention. Among the topics discussed were four central to the field: evolutionary and developmental influences on regenerative potential; the cellular sources of regenerating tissues; regeneration signals; and methods of enhancing regeneration. The developmental biology of regenerative events was represented in a spectacular array of organisms, from single-celled protists to plants, flatworms, fish, salamanders, mice and humans (Fig. 1). Myriad different experimental approaches were on display. The success of this meeting reinforces how the field of regeneration has renewed itself as a prominent domain in the minds of the scientific and general population.

Regeneration capacity across species and developmental stages

Regeneration is a useful trait. But why do many human tissues have such a limited capacity to regenerate? Have we lost the ability in our evolution, or did it never exist? How regeneration has been crafted or lost through evolution is often an informal late-night topic at regeneration meetings, with more bark than bite. At this conference, however, it took center stage. One way to think about this question is to examine regenerative capacity across many species. This was the norm in centuries past, but recent research has tended to focus on a small number of established models and to extrapolate from these. Presentations at this meeting delved into regeneration and its mechanisms in organisms simple and complex, and at different developmental stages and ages, to address the question of what controls regenerative capacity.

At the (relatively) simple end of the scale, Wallace Marshall (University of California, San Francisco, USA) showed findings that revive the study of the regeneration of *Stentor coeruleus*, a protist of 1-2 mm that was a popular model system for cell and developmental biology in the early to middle parts of the last century. After injury, these organisms reacquire lost pattern within their single cell, events that can now be dissected by RNA interference technologies. Moving into metazoa, Alexandra Bely (University of Maryland, USA) described regeneration in a group of annelids, the naids. Surprisingly, their capacity to regenerate after amputation varies widely even though these animals all reproduce by fission (Bely and Sikes, 2010). She also proposed a larger collaborative effort amongst the community to define and catalog regenerative potential among animals. We tend to think of mammals as being poorly able to regenerate, but Ashley Seifert (University of Florida, USA) described the remarkable regenerative capacity of the African spiny mouse (*Acomys*). The dermis and skin are extremely weak and tear easily away from their bodies, but the resultant large wounds are repaired remarkably well and regenerate hair follicles throughout the wound bed. Moreover, they can regenerate large hole punches in their ears much better than laboratory strains of mice. Thus, it is likely that unsuspected examples of regenerative capacity remain to be discovered, representing new model systems that will inform us of why regeneration does (or does not) occur.

Regenerative efficiency drops with age in mammals. This was illustrated by Amy Wagers (Harvard University, USA), who described multiple ways in which parabiotic sharing of circulation between young and old mice can have rejuvenating effects on regeneration in the older partner (Ruckh et al., 2012). Defining the circulating influences should provide new mechanistic views for regeneration as well as age-related disease. Anne Brunet (Stanford University, USA) presented the African killifish *Nothobranchius furzeri*, a species that needs a rapid life cycle to reproduce in ponds that dehydrate in the dry season, as a new model for the influences of age on regeneration. These animals have been isolated as short-lived (3 months) and long-lived (9 months) populations, and regenerate amputated fins like other teleost fish. Their accelerated lives, high regenerative capacity, and the opportunity for genome resources are all promising aspects of this system. By contrast, age might not play a major role in the ability of newts to regenerate lenses, according to Panagiotis Tsonis (University of Dayton, USA). He described experiments involving repeated lens removal (18 times) over a 16-year period, with no effects on regenerative capacity (Eguchi et al., 2011).

Many vertebrate species lose regenerative capacity as they mature developmentally. Juan Larrain (Universidad Catolica de Chile, Chile) described how stage 50-54 *Xenopus* tadpoles regain function after a severe spinal cord transection injury, a capacity that is soon lost in later stages. This system might help to define positive and/or negative influences on the outcome of spinal cord

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Fig. 1. A menagerie of regenerating systems discussed at the Keystone Symposium. From left to right: Annelid, mouse, newt, *Drosophila*, neuron, planarian, axolotl, killifish, *Xenopus*, *Arabidopsis*, human, zebrafish and cells, *Hydra*, *Stentor coeruleus*, African spiny mouse.

injury. Moving into humans, Bernhard Kühn (Children's Hospital Boston, USA) presented analyses of cardiomyocyte biology from an impressive set of healthy pediatric heart tissue. He presented evidence for cardiomyocyte mitosis and cytokinesis through late postnatal development, as well as mathematical modeling to implicate these events in cardiac growth. Such findings, together with the recent discovery of heart regeneration in neonatal mice (Porrello et al., 2011), suggest that the young human heart might have some capacity to regenerate after injury, a property thought to be absent in adult humans.

Origins of regenerating tissue

The development of new lineage-tracing tools, combined with the rise of stem cell biology, have led to recent discoveries of the cellular origins of regenerating tissues in many systems. In a wide range of contexts, resident stem cell populations provide a reserve that is activated by injury. Elsewhere, differentiated cells can act as source tissue. Work presented in Breckenridge balanced the importance of each and the mechanisms by which they maintain and regenerate tissues.

Stem cells must be maintained and activated appropriately throughout the lifetime of the organism. Roeland Nusse (Stanford University, USA) described how asymmetric Wnt signaling is important for controlling self-renewal of stem cells. He showed that the location at which an embryonic stem cell receives a bead-bound Wnt signal impacts the orientation of cell division. Furthermore, after cell division, the Wnt-tagged cell retains nuclear β-catenin as well as expression of pluripotency markers, in contrast to the cell without the Wnt signal. Valentina Greco (Yale School of Medicine, USA) also addressed the relevance of oriented division to cell fate, presenting two-photon imaging of stem cell mitoses in hair follicles of live mice. These visually beautiful data indicated that cells divide in stereotyped orientations during growth and that the mesenchyme surrounding the hair follicle is required for proper regeneration. In the same system, Elaine Fuchs (HHMI/Rockefeller University, USA) described the identification of hair bulge stem cells and subsequent work to define genes important for 'stemness'. One intriguing regulator was the Polycomb complex, which was found to repress stemness genes and de-repress commitment factors (Chen et al., 2012). In *Drosophila*, Allan Spradling (HHMI/Carnegie Institute, USA) applied lineage tracing and a Gal4/UAS variegation assay of epigenetic stability (Skora and Spradling, 2010) to the analysis of Lethal giant larvae-induced tumors in the ovarian follicle epithelium. De-differentiation and increased instability resulted from changes in the accessibility of dysplastic cells to specific stromal signals.

Stem cell biology was discussed in the settings of multiple other tissues. Christoph Lepper (Carnegie Institution for Science, USA) showed a series of Cre-based lineage-tracing/conditional gene knockout experiments in mice that revealed differential requirements for skeletal muscle satellite cells marked by expression of Pax7. These stem cells rarely participate during normal physiological aging or after exercise, but become activated upon severe injury and are robust contributors to myogenesis (Lepper et al., 2009; Lepper et al., 2011). Elly Tanaka (Center for Regenerative Therapies, Germany) described how spinal cord regeneration in axolotls involves neural stem cells that can be isolated, cultured into neurospheres, and transplanted to contribute multiple cell types to a regenerating spinal cord. Sean Morrison (HHMI/University of Texas Southwestern Medical Center, USA) presented experiments illustrating that an activated N-Ras mutation might have bimodal effects to promote both quiescence and competitive proliferation in subpopulations of hematopoietic stem cells. This suggested potential inroads to increasing long-term reconstitution without leukemogenic effects, a valued therapeutic goal.

The identification of new markers for intestinal crypt stem cells has stimulated a new understanding of how the intestinal epithelium is renewed (Barker et al., 2007; Sangiorgi and Capecchi, 2008; Tian et al., 2011). Linheng Li (Stowers Institute for Medical Research, USA) presented a cell surface marker-based method to facilitate the isolation of intestinal stem cells, with the goal of identifying stem cells with the highest developmental potential. The power of this approach was demonstrated by the isolation of single stem cells that generated enteriods and colonoids in culture, without the aid of support cells.

Regeneration can occur with or without a starting population of stem cells. For example, differentiated cells might de-differentiate into a stem cell state before regeneration can proceed. During root growth in *Arabidopsis*, new cells emerge from a well-characterized stem cell niche. Classic work had indicated that after removal of the stem cell compartment, the root retains the ability to rapidly regenerate, presumably by regenerating the stem cell niche from differentiating cells. However, the work of Kenneth Birnbaum (New York University, USA) showed that regenerative growth occurs before reconstruction of the stem cell center, and spared cells nearby can re-establish the lost cell types (Sena et al., 2009). This cellular recovery did not require re-establishment of stem cells, as it occurred in mutants that lacked a functional stem cell niche. Analogously, bone regeneration after fin amputation in zebrafish has been shown recently by several groups to rely on de-differentiation of resident osteoblasts (Knopf et al., 2011; Sousa et al., 2011; Tu and Johnson, 2011; Stewart and Stankunas, 2012). Kenneth Poss (HHMI/Duke University Medical Center) found using an inducible ablation method that there can be a second source of new bone that enables efficient regeneration when the starting osteoblast population is disabled (Singh et al., 2012).

Control of regeneration

For regeneration to occur, a tissue must sense the injury, initiate a regenerative response, and properly direct growth. Cell death has recently been shown to be a requirement for normal regeneration in multiple systems (Ryoo et al., 2004; Tseng et al., 2007; Li et al., 2010), and Brigitte Galliot (University of Geneva, Switzerland) described how mid-gastric amputation stimulates apoptosis in hydra polyps. These dying cells release signals, such as Wnt3, that stimulate the regenerative response (Chera et al., 2009). Interestingly, cell death appears to be limited to a population of cells arrested in the G1 phase of the cell cycle. Hydrogen peroxide production is another early signature of wounding, and was shown recently to attract immune cells to injury sites (Niethammer et al., 2009). Sandra Rieger (Mount Desert Island Biological Laboratory, USA) found that in amputated larval zebrafish fins, the regeneration of somatosensory neurons in the skin is guided by hydrogen peroxide (Rieger and Sagasti, 2011). Similarly, Jing-Wei Xiong (Peking University, China) implicated the localized production of hydrogen peroxide by epicardial cells in stimulating cardiomyocyte proliferation during heart regeneration in zebrafish.

Early signals for regeneration must connect to cell proliferation mechanisms. Jeremy Brockes (University College London, UK) and Alejandro Sánchez Alvarado (HHMI/Stowers Institute for Medical Research) examined the regulation and roles of classical mammalian 'tumor suppressors' in salamanders and planarians, respectively. In axolotls, p53 activity decreases during blastema formation and growth, before returning to normal levels during patterning of the regenerate. In newts, a similar mechanism applies, and the dominant-negative ΔNp73 isoform is also upregulated at the beginning of regeneration. In planarians, an archetypical model for stem cell-based regeneration, p53 or pTEN blockade in intact animals results in an initial phase of hyperproliferation, followed by proliferative failure and animal death (Oviedo et al., 2008; Pearson and Sanchez Alvarado, 2010). These manipulations also potently block regeneration after injury. Voot Yin (Mount Desert Island Biological Laboratory, USA) described how microRNAs are important for controlling cardiomyoctye proliferation after cardiac injury in zebrafish. He showed that miR-133 expression decreases in the heart after injury and, through transgenic manipulation, that levels of miR-133 have inverse effects on cardiomyocyte proliferation and regenerative ability (Yin et al., 2012).

Unsuspected interactions from nearby or distant cells can exert control over regeneration. Andras Simon (Karolinska Institutet, Sweden) described the spectacular regeneration of different types of neurons after injury to the adult newt brain, focusing mainly on dopaminergic neurons. These neurons regenerate from ependymoglial cells in the subventricular layer; once the appropriate number of dopaminergic neurons is restored, neurogenesis ceases (Berg et al., 2011). He proposed that this occurs through a feedback mechanism in which dopamine itself inhibits the formation of more dopamine-producing neurons. This has implications both for a control mechanism and for practical applications, such as deriving dopamine neurons in culture. Another example of neuronal control of regeneration was provided by Phillip Newmark (HHMI/University of Illinois). After head amputation in sexual planarian strains, the testes regress, and recover only after nervous system regeneration. Knockdown of the major prohormone convertase resulted in regression of reproductive tissues, and systematic characterization of planarian neuropeptides identified a neuropeptide Y homolog that links the nervous system to maintenance of the planarian reproductive system (Collins et al., 2010). He also described potential implications of these findings for understanding parasitic flatworms that infect hundreds of millions of people.

Applications

The ultimate applied goal of regeneration biology is to develop methods to improve regenerative capacity, facilitating disease prevention and recovery. This symposium ran concurrently with the Regenerative Tissue Engineering and Transplantation Symposium, enhancing the diversity of an already well-rounded group of participants. Many talks described the potential or ongoing application of recent discoveries toward therapies.

The application of human embryonic (ES) cells is limited by the availability of only a handful of cell lines. The closing keynote address by Susan Fisher (University of California, San Francisco, USA) described a new method for deriving lines from single, isolated blastomeres. These new lines possess all the properties of existing human ES cell lines, but have many additional characteristics that could make them especially useful for regenerative medicine therapies.

Manipulations that improve regenerative capacity in model systems provide tantalizing glimpses of future possible applications. Ömer Hidir Yilmaz (Massachusetts General Hospital/Whitehead Institute, USA) described how caloric restriction, known to enhance lifespan in many species, improves the capacity for in vitro enteroid formation by intestinal stem cells through a mechanism in which mTORC activity is reduced in Paneth cells (Yilmaz et al., 2012). Gufa Lin (University of Minnesota, USA) showed how a graft of regeneration-competent cells supplemented by a slow-release cocktail of factors, including Shh, Fgf10 and Thymosin Beta-4, could enhance the regeneration of amputated adult *Xenopus* limbs, which normally would only form a cartilage spike. Eric Lagasse (University of Pittsburgh, USA) is interested in cell therapies that provide function from ectopic sites, and has found promise in lymph nodes (Hoppo et al., 2011). He described how they have been able to transplant hepatocytes, pancreatic beta cell islets, and thymocytes into mouse lymph nodes, where these tissues grow, become vascularized and can serve functions of host organs.

High-throughput model systems give researchers the opportunity to use unbiased approaches to identify compounds and pathways that improve regeneration. Olov Andersson (University of California, San Francisco, USA) combined a system for inducible ablation of β cells in zebrafish larvae with a screen for small molecules that enhance or block the ensuing regeneration. Positive hits revealed that adenosine signaling increased β-cell proliferation and accelerated the restoration of normoglycemia, and these effects were also seen in a diabetic mouse model (Andersson et al., 2012). Finally, Michele de Luca (University of Modena and Reggio Emilia, Italy) presented an inspiring story of successful regenerative medicine, describing the growth and expansion of epithelial stem cells in vitro for skin grafts for severe burn victims. He also showed the long-term success in patients of transplanting corneal epithelium grown from cultured primary limbal stem cells, in which vision was recovered in a high percentage of tissue recipients (Rama et al., 2010). This work demonstrated the desperate need in clinics for regeneration-based discovery research and discovery-driven therapeutic solutions.

Conclusions

This meeting summary provides an overview of a tour de force of presentations representing a remarkably diverse canvas and topics. For those new to the field of regeneration, it may be easy to think

of how little we know of the essential mechanisms of regeneration. For those of us who have thought about questions in tissue regeneration for many years, it became clear at this meeting how far we have come.

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