Int. J. Dev. Biol. 62: 351-361 (2018)
https://doi.org/10.1387/ijdb.180084nv

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Lens regeneration: a historical perspective

M. NATALIA VERGARA[1*], GEORGE TSISSIOS[2] and KATIA DEL RIO-TSONIS*[,2]

[1]*Department of Ophthalmology, University of Colorado Denver School of Medicine, Aurora, CO, USA and*
[2]*Department of Biology and Center for Visual Sciences at Miami University, Miami University, Oxford, OH, USA.*

ABSTRACT The idea of regenerating injured body parts has captivated human imagination for centuries, and the topic still remains an area of extensive scientific research. This review focuses on the process of lens regeneration: its history, our current knowledge, and the questions that remain unanswered. By highlighting some of the milestones that have shaped our understanding of this phenomenon and the contributions of scientists who have dedicated their lives to investigating these questions, we explore how regeneration enquiry evolved into the science it is today, and how technological advances accelerated our understanding of these remarkable processes.

KEY WORDS: *lens, regeneration, transdifferentiation*

In memory of Panagiotis A. Tsonis (1953-2016).

Introduction

It is through the eyes of the curious that we have begun to uncover one of the most amazing mysteries in biology: regeneration. Take for instance the unraveling of eye tissue regeneration from Bonnet (1781), Colucci (1891) and Wolff (1895) to P.A. Tsonis (2016). These pioneers have brought into the realm of scientific enquiry matters that have entertained human imagination since the beginning of civilization; matters that used to belong to the domain of mythology, alchemy or metaphysics: Why can some animals regenerate body parts upon loss or injury? How does it happen? What are the sources? Can we harness that capacity to induce regeneration in normally non-regenerative tissues?

Thinking about regeneration today brings to mind the potential of regenerative therapies and the translational applications of stem cells. However, this has not always been the case. The history of regeneration research and thought has gone through several phases, each of them critical in challenging established dogmas and opening new ground for exploration. Regeneration has thus contributed along its history to our understanding of development, evolution and genetics.

Here we will provide a brief overview of some of the seminal works that paved the road to our current understanding of lens regeneration. This is by no means an exhaustive review of the field, which would be too broad for the purpose of this introductory article, but rather a selection of some of the key contributions that led to significant advances in our understanding of this remarkable process. A brief overview will be given on the history of regeneration in general for the purpose of placing the topic in the context of the collective thought and its implications at the time. For more thorough reviews the reader is directed to some remarkable works including Morgan, 1901; Dinsmore, 1991; Okada, 1996; Sanchez Alvarado, 2000; Sanchez Alvarado and Tsonis, 2006; Maienschein, 2009.

From Antiquity to the Middle Ages: the era of mysticism

We know that humans have entertained the idea of regenerating body parts for almost as long as we have a recorded history. Ancient Greek mythology told the story of Hydra, the monstrous multi-headed creature who was able to grow back two heads after losing one; and the story of Prometheus, condemned to watching his own liver be eaten by an eagle every day, only to regenerate it during the night. It would not be until the 20th century that the natural ability of the human liver to regenerate itself would be understood (Reviewed in Tsonis, 1996; Sanchez Alvarado, 2000).

The first recorded observations of regeneration can be attributed to Empedocles (490-430 B.C.) and to Aristotle (384-322 B.C.), who commented on the ability of lizards to regenerate their tails. But there would be a long gap until animal regeneration would be "re-discovered" and studied as a natural phenomenon. In Medieval Europe, regeneration would fall into the domain of alchemy, being

Abbreviations used in this paper: dpl, days post-lentectomy; LEC, lens epithelial cell; PEC, pigmented epithelial cell.

***Address correspondence to:** M. Natalia Vergara. Department of Ophthalmology, University of Colorado School of Medicine, 12800 East 19th Avenue, Mail Stop 8311, RC-1 North, Room 5106, Aurora, CO 80045, USA. Tel: 303.724.3579. Fax: 303.724.5270. E-mail: natalia.vergara@ucdenver.edu - https://orcid.org/0000-0003-4545-8181 or Katia Del Rio-Tsonis. Department of Biology, Miami University, Oxford, OH, 45056-1400, USA. Tel: (513) 529-3128. Fax: (513) 529-6900. E-mail: delriok@miamioh.edu - https://orcid.org/0000-0002-3051-6716

Submitted: 4 March, 2018; *Accepted:* 5 March, 2018.

ISSN: Online 1696-3547, Print 0214-6282

linked to the idea of the "Elixir of Life."

It was not until the eighteenth century that there would be a resurgence of natural enquiry, beginning in the form of a cataloguing of natural observations.

The 18th Century: escaping alchemy

What followed was a sudden upsurge in interest in the study of regeneration, which would carry important scientific and philosophical implications. Reaumur could perhaps be considered the father of the field. In 1712, he reported that crayfish had the ability to regenerate a new limb upon amputation (Dinsmore, 1991). He was followed by Abraham Trembley, who discovered in 1740 that a microscopic animal was capable of regenerating its head after amputation. This ability and its morphological appearance, with tentacles protruding out of its head, prompted Trembley to name this organism after the mythical Hydra. In 1745, Charles Bonnet added annelids to the now growing list of animals with regenerative capabilities, and in 1766, Peter Simon Pallas described the remarkable regenerative properties of a then new group of animals that we now know as planarians (Reviewed in Elliott and Sanchez Alvarado, 2013).

Insights on vertebrate regeneration came from Lazzaro Spallanzani's work in 1768. He described salamanders as potent regenerators, capable of regrowing their limbs, tails and jaws upon amputation (Spallanzani, 1768; reviewed in Tsonis, 1996; Tsonis and Fox, 2009). The systematic experimental approach taken by Spallanzani and some of his predecessors transformed natural enquiry into an experimental science and established methods that could be followed and reproduced by other experimentalists.

This new approach, combined with the renewed interest in exploring these biological phenomena, stirred significant philosophical arguments as naturalists strived to reconcile their observations with the strong religious beliefs that influenced scientific interpretations in the seventeenth and eighteen centuries. Thus, in the emerging field of embryogenesis two different paradigms arose to attempt to provide an explanation of animal development and regeneration, which were seen as closely linked: preformation vs. epigenesis. Preformationism was the most generally favored position, which contended that the sperm or egg already contained a miniature animal (called a "homunculus" in the case of humans), which subsequently expanded in size over the course of development. This explanation allowed for the possibility that all generations were established within one another at the time of creation, but it also implied that all body structures should be present from the beginning and subsequently just get enlarged. The opposing view, epigenesis, stated that animals were gradually built during development, starting from undifferentiated embryonic cells; thus, new structures would arise by progression through a number of different stages (reviewed in Dinsmore, 1991; Okada, 1996). The new studies on regeneration began to cast doubts on the preformationist hypothesis (reviewed by Dinsmore, 1991; Elliott and Sanchez Alvarado, 2013). Moreover, in the 18th century context, these studies posed another major philosophical dilemma: that of the indivisibility of the soul, since if some animals could regenerate their amputated heads or even the entire organism from two segments, then where did the soul reside?

The first report on eye tissue regeneration was recorded in Bonnet's Oeuvres d'histoire naturelle et de philosophie (1781) where he described grossly the partial dismemberment of a salamander's eye and the surprising appearance of the complete eye several months later. Philippeaux (1880) confirmed Bonnet's observations assuring that part of the eye must be left behind in order for it to regenerate (described in Morgan, 1901).

The 19th Century: the triumph of the scientific method

It would take another 100 years after the early reports on salamander eye regeneration for researchers to pursue more detailed studies on the regeneration of the lens. In 1891 Colucci reported that newts were able to regenerate their ocular lenses upon removal even at adult stages; and in 1895 Wolff published his independent studies where he confirmed that the cellular source for this regeneration was the pigmented epithelial cells (PEC) of the iris (Wolff, 1895). This was particularly intriguing considering that these cells are not involved in the developmental origin of the lens. Interestingly, this experiment was an attempt by Wolff to criticize Darwin's theory of evolution. This phenomenon would eventually be known as Wolffian regeneration (Dinsmore, 1991; Call *et al.*, 2005; Henry and Tsonis 2010).

The 20th and 21st Centuries: unraveling the mechanisms

In 1901 Thomas Hunt Morgan, the American embryologist who later went on to win the Nobel Prize for his work on the role of chromosomes in inheritance, published his compendium on "*Regeneration*", where he summarized and critically evaluated the works of the previous century and placed regenerative biology in the context of development. Morgan viewed regeneration as a fundamental developmental process that is widespread in the animal kingdom, rather than a simple case of adaptation, and made his case by working on a wide variety of organisms, as at the time, studies were considered more robust if they could be repeated in different species rather than privileging results from a narrow selection of them (Morgan, 1901; Reviewed in Sunderland, 2010).

Morgan can be credited with establishing the denominations for the general subdivision of regeneration into two main categories that still hold today: epimorphosis and morphallaxis. Epimorphosis refers to a regenerative phenomenon in which the development of the new part involves cell proliferation, whereas morphallaxis refers to regeneration resulting from the remodeling of existing material without proliferation (Reviewed in Sunderland, 2010). Lens regeneration in newts is a classic example of epimorphosis.

The Newt eye

In the 1930's Tadao Sato traveled from Japan to Freiburg, Germany, to study embryology under the mentorship of Nobel Laureate Hans Spemann and Otto Mangold. During this time, he became interested in the process of lens regeneration in newts, and produced some intriguing insights into the cellular mechanisms behind this phenomenon. He discovered that only the PEC from the dorsal part of the iris and not the ventral part of it were able to produce a regenerated lens (reviewed in Okada, 1994; Yasuda, 2004). Upon his return to Japan, Sato was appointed Professor at Nagoya Imperial University, where he mentored Goro Eguchi, who would go on to advance the field of lens regeneration research in the coming years (Okada, 1994; Yasuda, 2004).

In 1940, Stone and Sapir at Yale University had recognized that the mechanism of lens regeneration in urodele amphibians such as newts involves the process of transdifferentiation, that is, the conversion of one differentiated cell type into another (Stone and Sapir, 1940). Eguchi explored this process using the then cutting-edge technology of electron microscopy, providing a definitive histological and temporal characterization of newt lens regeneration at the cellular level (Eguchi 1963; 1964). He determined that after lens removal, the PEC of the dorsal iris dedifferentiate, losing the characteristics that define their cell type identity such as pigmentation. The iris PEC re-enter the cell cycle about 4 days post-lentectomy (dpl) when they also start to depigment. At 8-10 dpl, the depigmented cells at the tip of the dorsal iris proliferate to form a vesicle that contains an inner and outer layer. At days 12-16, the cells of the inner layer elongate and begin to differentiate into primary lens fiber cells and synthesize crystallin proteins. A second peak of proliferation also takes place at this time. Twenty-five dpl, the regenerated lens consists of a layer of lens epithelial cells in the anterior surface and correctly organized lens fiber cells on the posterior part (Eguchi, 1963; 1964; reviewed in Yamada 1977; McDevitt and Brahma, 1982; Tsonis, 2000; Tsonis *et al*., 2004a; Del Rio-Tsonis and Eguchi, 2004; Call *et al*., 2005; Sousounis *et al*., 2014a). An illustration on the process of newt lens regeneration is depicted in Fig. 1A.

Further insights into the regulation of this phenomenon were gathered using classic transplantation approaches. When newt dorsal irides were transplanted into lentectomized eyes of non-regenerating salamanders, they were able to form a lens, whereas regeneration did not take place if the transplantation was performed into the body cavity, suggesting that other extrinsic factors might influence the regenerative capacity of the tissue (Ikeda, 1934, 1935, 1936; Amano and Sato, 1940; Reyer, 1953, 1954, 1956; Stone, 1958a). The neural retina was proposed as a source of permissive factors, as its presence was able to rescue lens regeneration in the previous experimental settings, while separating the neural retina from the iris prevented lens regeneration (Stone, 1958a,b). On the other hand, the presence of a lens had the opposite effect on regeneration. If the lens was removed and another lens was replaced near the dorsal iris, even when this lens was derived from a different salamander species, it was able to inhibit regeneration. The same was true if a dorsal iris explant was placed in the anterior part of a lens-containing eye (Stone, 1953; Eguchi, 1961; Reyer, 1961). In 1952, Stone showed that a lens is only inhibitory if it contains its capsule, a transparent basement membrane structure that completely envelops the lens. Therefore, regenerating lenses do not have inhibiting properties until the point when the lens capsule is made, which coincides with the time when the regenerating lens detaches from the dorsal iris, usually 25-30 dpl (Stone, 1952; reviewed in Thornton, 1956).

Interestingly, Eguchi's group discovered that *in vitro*, both the dorsal and ventral iris can form lentoid bodies, rather amorphous structures containing lens fibers, suggesting that the newt's ventral iris PEC have the potential for transdifferentiation, but that this is not normally permitted *in vivo* (Eguchi *et al*., 1974). The challenge was to induce the ventral iris to regenerate a lens. Cleverly, Eguchi and Watanabe (1973) successfully induced lens regeneration from the ventral-incompetent iris by exposing the lentectomized eye to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a potent carcinogen (Fig. 2). Surprisingly, this ventral PEC reprogramming was maintained through a subsequent lentectomy (Eguchi and

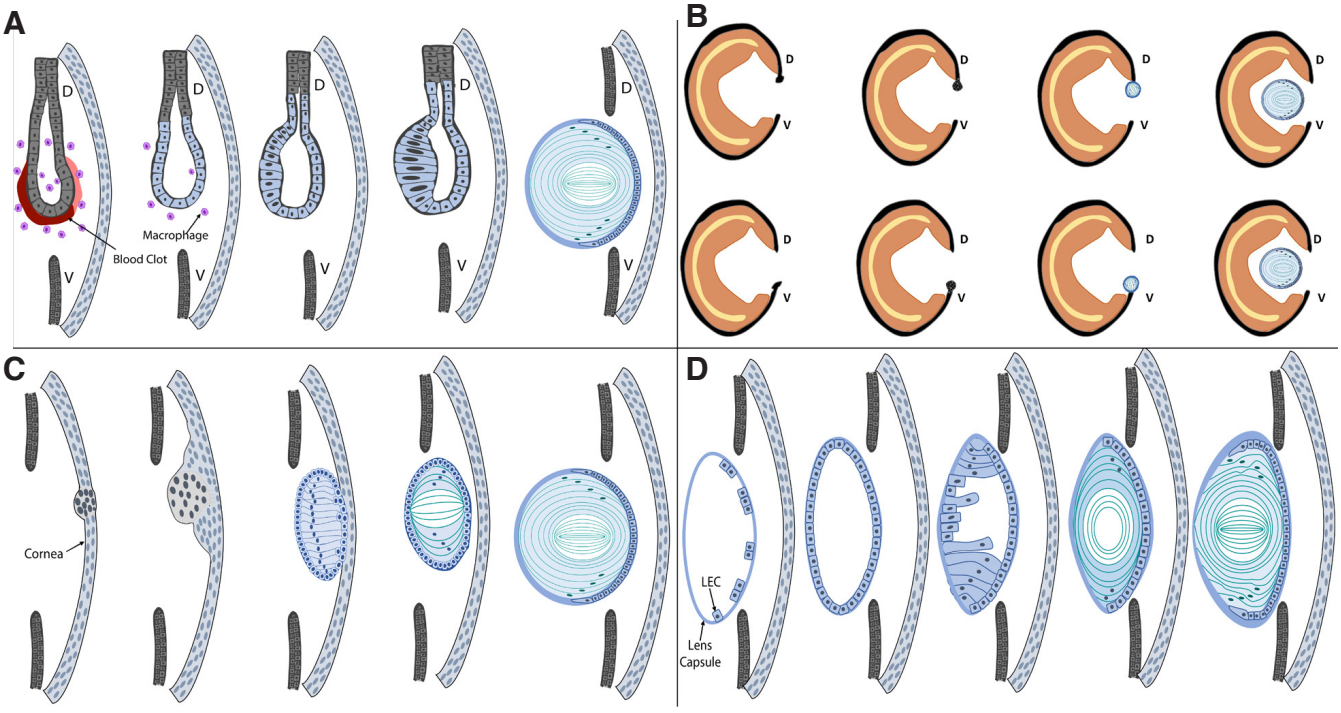

Fig. 1. Lens regeneration in different species. (A) *A series of illustrations depicting newt lens regeneration. Adult newts regenerate their lenses through transdifferentiation of cells at the edge of the dorsal iris, but not the ventral part of this tissue.* **(B)** *The axolotl larvae is able to regenerate its lens from either dorsal or ventral iris.* **(C)** *Lens Regeneration in* Xenopus *takes place from the cornea.* **(D)** *Mammals can regenerate their lens via a regrowth of the lens epithelial cells left inside the capsule. D, dorsal iris; V, ventral iris; LEC, lens epithelial cell.*

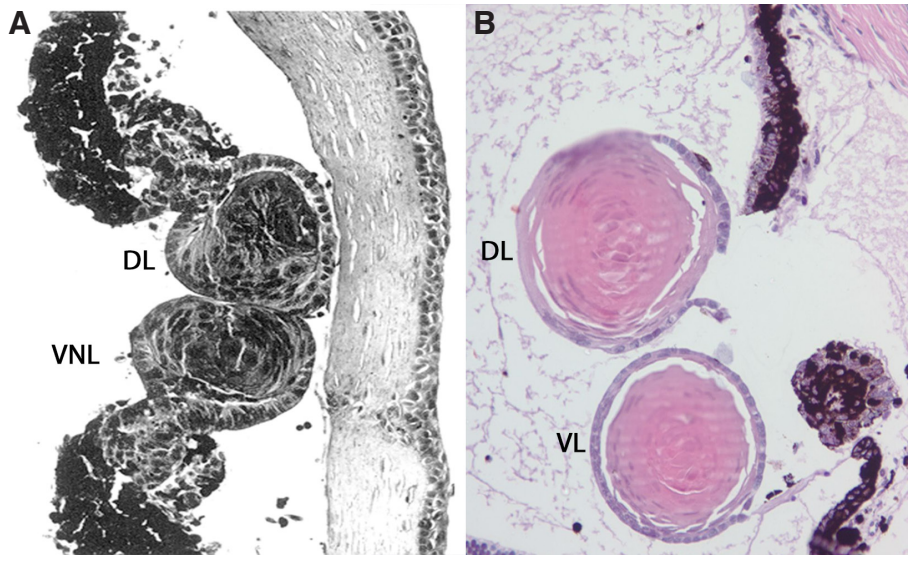

Fig. 2. Induction of ventral regenerates. *First cases of lens induction from ventral iris PEC:* **(A)** *by exposing lentectomized newt eyes to the potent carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and* **(B)** *by inhibiting the BMP pathway with chordin in ventral iris PEC that were implanted into a lentectomized newt eye. DL, dorsal iris regenerate; VNL, ventronasal lens regenerate; VL, ventral lens regenerate (From Eguchi and Watanabe, 1973 and Grogg* et al.*, 2005).*

Watanabe, 1973). As impressive as this task was, a molecular path for the induction of lens regeneration is still to be uncovered.

New technologies bring new opportunities

The field of lens regeneration has evolved as technological advances and conceptual discoveries have allowed for a deeper understanding of this extraordinary process. The description of this process has moved from a morphological and histological one to a cellular and molecular inquiry (reviewed in Del Rio-Tsonis and Tsonis, 2003; Del Rio-Tsonis and Eguchi, 2004; Tsonis *et al.*, 2004a; Barbosa-Sabanero *et al.*, 2012 and Sousounis *et al.*, 2014a).

Panagiotis A. Tsonis, the first Greek citizen to receive a PhD degree from a former Japanese Imperial University, was introduced to the study of regeneration through the mentorship of Goro Eguchi. He would eventually become widely recognized not only for advancing our understanding of regeneration in vertebrates, but also for his significant efforts in bringing the newt, an animal model with outstanding regenerative capacities but challenging genetics, into the molecular era (Singh, 2016).

At the turn of the century, regenerative biologists were still puzzled by the question of why the newt's dorsal and ventral iris, two sides of the same tissue, have such different regenerative potential.

Tsonis, then a Professor at the University of Dayton, saw in this problem an opportunity to use the newly available molecular tools to perform comparative studies between dorsal and ventral newt iris PEC, and thus began the quest to dissect the differential transcriptomic profiles and regulatory mechanisms in these tissues. If the right information could be gathered using this paradigm, it would provide the key to unlocking the regenerative capacity in normally non-regenerative tissues such as the newt ventral iris or even in other species.

Through his work and that of his collaborators, a regulatory network began to unravel. Genes that were known to be important in lens development became natural candidates for studying their potential involvement in regeneration. Thus, it was discovered for example that Pax-6, a master regulator of lens development involved in different aspects of this process from lens placode specification to lens fiber differentiation (Cvekl and Ashery-Padan, 2014), was re-expressed in newts during the dedifferentiation of the PEC and subsequent lens regeneration (Del Rio-Tsonis *et al.,* 1995; Mizuno *et al.*, 1999a; Madhavan *et al.*, 2006). Other cell-intrinsic factors that were identified in this process included hox genes and Prox-1 (Jung *et al.,* 1998; Del Rio-Tsonis *et al.,* 1999; Mizuno *et al.*, 1999a).

The search for extrinsic regulatory factors led to the identification of fibroblast growth factors (FGFs). FGFs and their receptors are expressed during lens regeneration, and exogenous FGF administration is able to induce the formation of a second lens, but only from the dorsal iris (McDevitt *et al.*, 1997; Del Rio-Tsonis *et al.*, 1997; Hayashi *et al.*, 2004). Moreover, inhibition of FGF receptor signaling alone can abolish lens regeneration (Del Rio-Tsonis *et al.*, 1998; Hayashi *et al.*, 2004). Interestingly, adding FGF2 to an intact eye induces the dorsal iris to make a lens (Hayashi *et al.*, 2004). Therefore, it is suggested that FGFs secreted by the neural retina might thus be the factors responsible for the inductive effect of the retina on lens regeneration. Other important factors found to be involved in lens regeneration include retinoic acid (Tsonis *et al.*, 2000), complement components (Kimura *et al.*, 2003); Wnt (Hayashi *et al.*, 2006); and hedgehog (Tsonis *et al.*, 2004b) though none of these was able to induce regeneration from the ventral iris *in vivo*.

Despite these advances, many questions remained unanswered: What initiates cell cycle re-entry? Are these activities restricted to the dorsal iris? Are cell cycle re-entry and the process of dedifferentiation coupled? Insights into these questions came from several groups. Work from Brockes' group showed that the dorsal iris expresses Tissue factor (F3; also known as coagulation factor 3, tissue factor), which in turn activates the thrombin pathway, creating a fibrin clot within 20-30 minutes after lens removal exclusively in the dorsal iris. Fibrin can then recruit marcophages and FGFs to initiate the process of dedifferentiation and cell cycle re-entry (Imokawa and Brockes, 2003; Godwin *et al.*, 2010). Interestingly, the ventral iris PEC appear to also reprogram and enter the cell cycle; however, these cells fail to contribute to lens replacement (Fig. 1A). Work by Maki *et al.,* (2007) showed that nucleostemin, a nucleolar stem cell marker, is expressed in both dorsal and ventral iris PEC as early as 2 dpl, way before the entry into the cell cycle. Other stem cell markers such as pluripotency factor Sox-2 is also upregulated before cell cycle entry peaking at 2 dpl in both parts of the iris, whereas cMyc is clearly upregulated at 8 dpl in dorsal and ventral irides correlating with cell cycle entry (Maki *et al.*, 2009).

Ultimately in 2005, Tsonis and his group published a seminal work that achieved for the first time the induction of lens regeneration from the newt's ventral iris by known factors (Grogg *et al*., 2005): this was accomplished by two different experimental strategies: i) overexpression of the transcription factor six-3 with concomitant retinoic acid treatment, and ii) inhibition of the BMP pathway, which lies upstream of the pax-6/six-3 regulatory loop (Fig. 2). Interestingly, this study also highlighted the complexities of a biological system, as six-3 was normally expressed in both the dorsal and the ventral iris, yet the lentectomy induced a higher relative increase in expression levels in the dorsal side, and that seemed to confer the regenerative competency. This lead to the hypothesis that for the iris to become competent for regeneration, levels of expression of regulatory genes must be elevated above established thresholds (Grogg *et al.,* 2005; reviewed in Tsonis, 2006). Subsequently, Hayashi *et al.,* (2006) showed that Wnt2b and its receptor Frizzled-4 are mostly upregulated in the dorsal iris at later stages of lens regeneration, and that ventral induction was also possible when ventral iris explants were treated with FGF2 and Wnt2b.

Further understanding of gene expression differences during this regenerative process was obtained by analyzing patterns of histone modifications. Such patterns suggest a general activation state in both irides during the dedifferentiation process, while the repressive mark H3K27me3 is uniquely retained in the ventral iris at this stage (Maki *et al*., 2010a). It is interesting to note that the linker histone B4 (H1foo), associated with germ cell packaging, is a key repackaging histone during the process of lens transdifferentiation (Maki *et al*., 2010b). This may reflect the extended epigenetic and transcriptional changes required for the successful completion of iris to lens transdifferentiation.

The surge of the "Omics" era

A series of groundbreaking work was unleashed with the advent of next-generation sequencing and the availability of databases, microarrays, RNA-seq and proteomic approaches. The Tsonis' lab was a pioneer in this feat. While the newt genome is gigantic, about 10x larger than the human genome, this did not stop these researchers from aggressively pursuing transcriptomic and proteomic efforts in collaboration with Thomas Braun and Mario Looso, to ultimately contribute to the first *de novo* assembly of the North American newt *Notophthalmus viridescens* transcriptome (Looso *et al*., 2013). Tsonis also collaborated with Randal Voss and Jeramiah Smith to initiate efforts to sequence the newt genome creating the first linkage map (Keinath *et al*., 2017). Early efforts using microarrays to compare the initial stages of lens regeneration (up to 5 dpl), pointed to the already suspected outcome of similar patterns of gene activation in both dorsal and ventral irides during the stages prior to cell cycle entry (1-3 dpl), indicating activation of redox homeostasis, DNA repair programs and matrix remodeling enzymes. Cell cycle genes were active at the later stage of 5 dpl (Sousounis *et al*., 2013a).

Results from the proteomics and RNA-seq efforts opened up a sea of data, highlighting again that both the dorsal and ventral irides differ mostly on the amount of transcripts rather than on their uniqueness. These studies were done collecting RNA from two critical time points: 4 and 8 dpl, emphasizing cell cycle entry, mitotic differential activities as well as the dedifferentiation processes. Indeed, the most relevant functional groups of genes regulated in the dorsal iris were cell cycle, immune response, and cytoskeletal genes, while in the ventral iris there was an intriguing enrichment of transposon transcripts. One unique observation was the differential expression of dorsal-ventral specific genes *Tbx5*, highly expressed in the dorsal iris, and *Vax2*, highly expressed in the ventral iris (Sousounis *et al*., 2013b; Sousounis *et al*., 2014b). Testing the ability of these transcription factors to regulate the fate of the dorsal or ventral iris can now be addressed more efficiently by using CRISPR/Cas9 technology in combination with an efficient *in vitro* dorsal-ventral iris culture system (Grogg *et al*., 2005) that can be scored by the presence or absence of lens organoids (Hoffmann *et al*., 2014) (Fig. 3). Another insightful data obtained in those studies was the clear differential distribution of cell guidance molecules in the dorsal and ventral irides. For example, Ephrin-B2 was found to be highly expressed in the dorsal iris (>16 times) and its receptor, EphB, in the ventral iris (>2-5 times); whereas Netrin-1 was highly expressed in the ventral iris (>32 times) and its receptor UNC-5B in the dorsal iris (>16 times) (Sousounis *et al*., 2013b). These molecules have been associated with cell repulsion, establishment of boundaries and cell communication (Keleman *et al*., 2001; Genander *et al*., 2010). Therefore, Ephrin-B2/EphB and Netrin-1/UNC-5B signaling as means of repulsion between the dorsal and the ventral iris, if proven, will create a new paradigm and will shed light on unsuspected players in lens regeneration.

Defeating aging during regeneration

In an epic collaboration between Tsonis and his former mentor Goro Eguchi (or Sensei as he used to call him; Fig 4), the ability of the newt to undergo repeated regeneration was evaluated with the goal to once and for all settle if the newt lost regenerative abilities with repeated lens removal and regeneration, as well as with increased age. To their amazement, their 18 year-long experiment demonstrated that the outstanding capacity for lens regeneration in newts is maintained even at old age, and that newts that had undergone lens regeneration up to 19 times were still able at

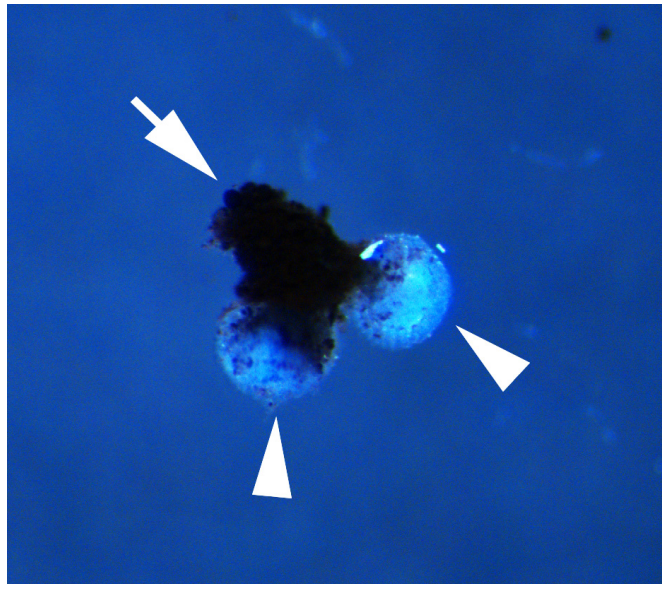

Fig. 3. *In vitro* lens engineering. *Phase contrast image along with Hoechst nuclear stain of a Matrigel-cultured dorsal iris PEC aggregate (arrow) with two lens-like structures (arrowheads). (From Hoffmann* et al.*, 2014).*

around 30 years of age to make lenses that were structurally and transcriptionally similar to young newt lenses (Eguchi *et al.*, 2011; Sousounis *et al.*, 2015) (Fig. 5). This was counter to the commonly held belief that regeneration would become less efficient over time or with advanced age even in these animals. Using RNAseq analysis, they further verified that these repeatedly regenerated lenses from aged newts had a robust transcriptomic program that was not disturbed by age or repeated insults. Comparable tail tissues from the same aged animals that never underwent regeneration showed a transcriptomic program in line with an aged tissue such as de-regulation of electron transport chain genes (a defacto sign of aging). As better said by Tsonis in their concluding remarks: *"These observations provide in our opinion strong evidence that a robust transcriptional program ensues after an insult to guarantee that the regenerative ability in newts will not be thwarted with age"* (Sousounis *et al.*, 2015). Beguilingly, Yun *et al.* have recently described that newts possess a unique ability to clear out senescent cells using macrophages during limb regeneration. It was suggested that this ability equips these amphibians with their remarkable capacity to regenerate their limbs even at old age (Yun *et al.*, 2015). It is possible that this same mechanism empowers newts for repeated lens regeneration in aged animals.

The other regenerators …

Lens regeneration in the axolotl - not all salamanders are made equal…

Stone (1967) described that not all salamanders are able to regenerate their lens from the dorsal iris. Among those with lower regenerative abilities are members of the genus *Amblystomidae*. Indeed lens regeneration studies in adult axolotls (*Amblystoma mexicanum)* have been performed only to find that they lack that capacity. Axolotls have been used since as a comparative organism to test inductive abilities: from early transplantation experiments to modern "omics" data analysis (Gross, 1969; Grogg *et al.*, 2005; Sousounis *et al.*, 2014c). Recently Brockes' group showed that the adult axolotl not only did not express Tissue factor in the dorsal iris, but that it was unable to undergo thrombin activation and thus failed to initiate the process of lens regeneration from the iris PEC (Godwin *et al.*, 2010).

Fig. 4. Two Regeneration Giants planning the last of the lens regeneration aging experiments (Japan 2012). *Panagiotis Tsonis (left) and Goro Eguchi (Right).*

Even though the newt is the "champion of the champions" in regenerative capabilities, it is not an ideal model organism for genetic studies as it is difficult to rear in the lab and its reproductive cycle is quite long. Few labs worldwide have succeeded in rearing newts and establishing transgenesis/CRISPRCas as an approach for gene manipulation (Casco-Robles *et al.*, 2011; Hayashi and Takeuchi, 2016; Elewa *et al.*, 2017). An alternative approach is to search for an amenable organism for genetic manipulations that could have a potential to regenerate, maybe at an earlier stage, and voila: an axolotl larvae! (Suetsugu-Maki *et al.,* 2012; Khattak *et al.*, 2014). Tsonis' lab was able to pinpoint a small window of time in which the axolotl is able to regenerate its lens: starting at stage 44 (hatching stage), time at which the lens of the eye is fully developed (Armstrong and Malasinski, 1989) and up to 14 days post-hatching. The lens of the axolotl at these stages regenerates from the iris, but not necessarily from the dorsal or the ventral part of it. Interestingly, regeneration occurs quite fast, showing a lens vesicle within 6 hours (Fig. 1B) (Suetsugu-Maki *et al.*, 2012). Microarray analysis comparing the lens regeneration competent and non-competent stages revealed that the competent iris was enriched with electron transport chain, transcription, metabolism, and cell cycle-related genes, whereas the non-competent iris transcriptome was enriched in cell differentiation and tissue maturation genes, patterns associated with aging differences (Sousounis *et al.*, 2014c). In all, the axolotl larvae has opened a door for further explorations unto the mysteries of Urodelian lens regeneration, and with the recent sequencing and assembly of the axolotl genome (Nowoshilow *et al.*, 2018), this model is sure to become an invaluable resource for this field of research.

Lens regeneration in the frog

In 1963, Freeman expanded our knowledge of animal species capable of regenerating their ocular lenses when he published his findings on lens regeneration in frogs. This type of regeneration differs in some ways from that of Wolffian lens regeneration observed in newts (Reviewed in Henry and Tsonis, 2010). The source of lens regeneration in frogs is the corneal epithelium, and it is stimulated by factors secreted by the retina (Freeman, 1963; Filoni *et al.*, 1981, 1982, 1983). It is important to point out that this phenomenon occurs during larval stages, and that the success and extent of lens regeneration decreases as the larvae approach metamorphosis (Freeman, 1963; Filoni *et al.*, 1997; reviewed by Henry and Tsonis, 2010; Tseng, 2017).

It is not clear, however, whether lens regeneration in *Xenopus* involves cellular dedifferentiation, as the cornea is not yet fully differentiated at larval stages (Fig. 1C) (Yamada, 1982; McDevitt and Brahma, 1979; Bosco, 1988; Henry, 2003). Another difference with the Urodelian paradigm is that the cornea and lens share the same embryonic origin, raising the possibility that the regenerative capacity in this case might be linked to an extended period of competence of the larval surface ectoderm to respond to lens inducing signals (see Schaefer *et al.*, 1999; Mizuno *et al.*, 1999b, 2005; Henry *et al.*, 2002; Henry, 2003; Cannata *et al.*, 2003; Malloch *et al.*, 2009; Filoni, 2009). Moreover, Kha *et al.,* (2018) have

shown that upon complete surgical removal of the developing *Xenopus* eye at the tailbud stage, a new eye is able to regrow, replacing the missing structures including the lens. Even though the source of the regrown lens has not yet been characterized, it is likely that it could be the remaining surface ectoderm, which would also indicate that significant plasticity is still in place at this stage of development. Interestingly, Perry *et al.,* (2013) have recently described that the cornea epithelium in the *Xenopus* tadpole expresses pluripotency factors including *sox2, p63, oct4, c-myc* and *klf4*, suggesting the intriguing possibility that the source of lens regeneration in these animals might be oligopotent epithelial stem cells present in the basal corneal epithelium. Nevertheless, adult frogs *in vivo* are unable to regenerate whole lenses except when the lens capsule is left intact and lens epithelial cells attached to it provide a source for re-growth, as is the case in mammals (see next section; Call *et al.,* 2004; Yoshii *et al.,* 2007; Lin *et al.,* 2016).

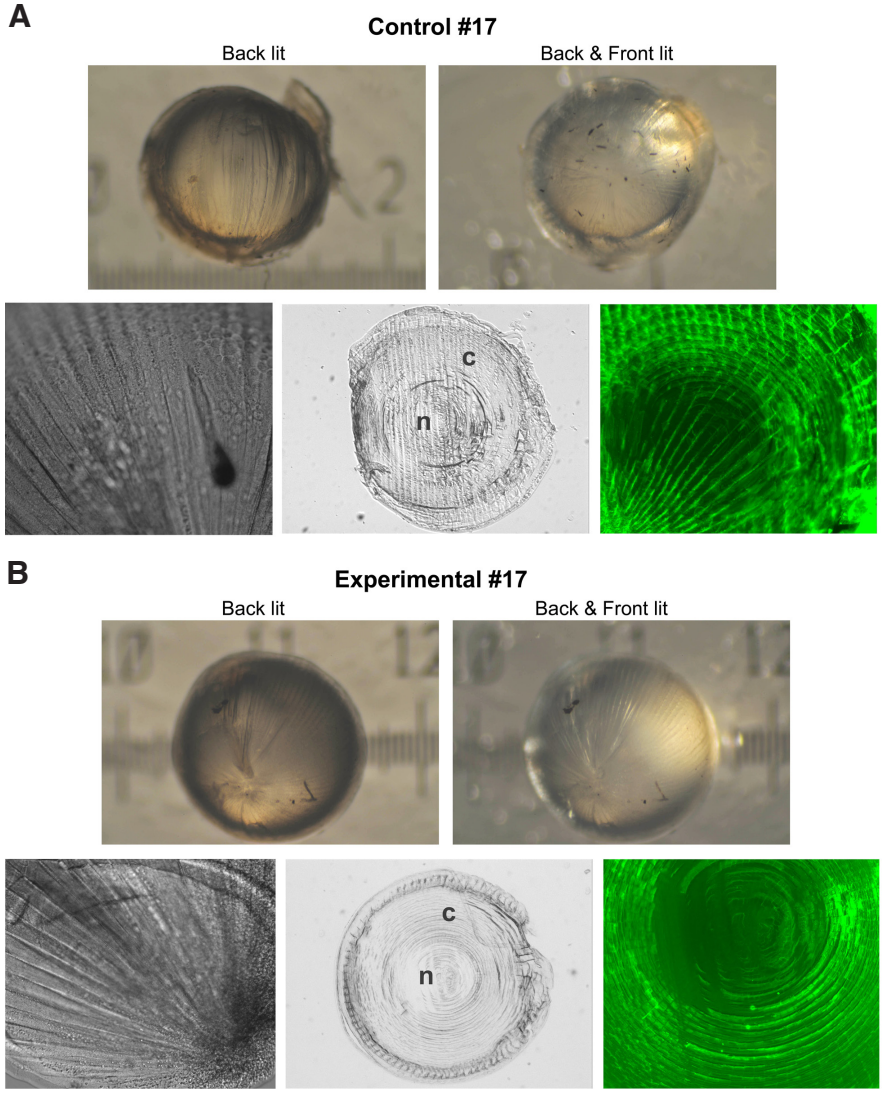

Fig. 5. Repeated regeneration. (A) *Intact lenses (Control no.17) from newts that never underwent regeneration have similar size, lens fiber arrangement and morphology, crystalline expression and transparency than lenses that* **(B)** *regenerated 17x (Experimental no. 17). n, nucleus, where primary fibers are present; c, cortex, where secondary fibers are present. From Eguchi* et al.*, 2011.*

However, Hamilton and Henry (2016) have recently shown that post-metamorphic corneal *in vitro* explants can initiate the formation of lens cells via the activation of limbal stem cells and transit amplifying cells of the cornea.

Lens regeneration in mammals

Mammalian lens regeneration has been described since the 19th century, but in contrast to the robust regenerative animal models that we have discussed so far, the regeneration process in mammals is quite different. Mammals are only able to regenerate their lens when the lens capsule is left behind during surgery, and the process does not involve transdifferentiation. The source of lens regeneration in these animals is the lens epithelial cells that remain adhered to the capsule during surgery and cannot be completely removed (Fig. 1D). Therefore, removal of the capsular bag will result in the absence of regeneration (Gwon *et al*., 1989, 1990; Call *et al*., 2004; Reviewed in Gwon, 2006; Tsonis, 2006).

The first account of spontaneous lens regeneration after removal of the contents of the lens capsule in mammals can be attributed to Cocteau and D'Etoille, who in 1827 described this phenomenon in New Zealand albino rabbits (Reviewed in Gwon, 2006). Several other research groups confirmed those findings during the following century, and even though rabbits remained the preferred animal model, investigations were further expanded to include cats, dogs, sheep, cow, and Guinea pigs, with variable success (Pettit, 1963; Gwon, 2006; and references therein). Of note, this process was reported to be age-dependent, achieving a higher degree of regeneration and at a faster rate in younger animals (Millot, 1872; Gwon *et al*., 1992; Gwon, 2006 and references therein).

In most of these reports lens regeneration is achieved to an imperfect degree. The regenerated lenses contain crystallins and other lens proteins, but they usually display defects including irregular lens fiber shape and alignment. These defects are affected by the surgical incision and adhesion between the anterior and posterior capsule (Gwon *et al*., 1990).

In 2004, Tsonis' group characterized a model of lens regeneration in the mouse. The mechanism of regeneration in this case was similar to that of other mammals, but this model provided the opportunity to take advantage of the broader availability of molecular and genetic tools available in mice to probe into the regulatory mechanisms that control this process (Call *et al*., 2004). At the initial stages of regeneration, epithelial to mesenchymal transition (EMT) of lens epithelial cells was the prevalent mechanism, suggesting a wound healing response. This mechanism is similar to that of posterior capsule opacification as observed in humans after cataract surgery. By 20 days post-lentectomy signs of EMT had diminished considerably, being replaced by a

lens differentiation process that resembled many aspects of its developmental counterpart (Call *et al.*, 2004). The first lens fibers could be observed within two days post-lentectomy, and by 30 days the regenerated lens had achieved at least half the size of the intact one, displaying an established equator with well differentiated bow regions (Call *et al.*, 2004). Similar studies were also performed in rats by Lois *et al.,* in 2003.

In 2006, Tsonis' group undertook a microarray-based study using the mouse model with the goal of identifying regulatory elements involved in the different stages of this process. The global gene expression and clustering analysis confirmed the expression of known modulators of EMT at the early stages of regeneration, including the elevation of transcripts involved in response to injury and extracellular matrix remodeling, and the onset of a lens fiber differentiation program at later stages (Medvedovic *et al.*, 2006).

Significantly in 2016, Lin *et al.,* developed a new capsulorhexis method for cataract surgery that decreases the size of the wound and shifts its location from the central visual axis to the periphery (Tan *et al.*, 2017), with the goal of improving visual axis transparency and maximizing the preservation of lens epithelial cells with regenerative potential. They tested this method in rabbits and macaques, achieving the formation of a biconvex lens in the latter at five months post-surgery (Lin *et al.*, 2016). An initial level of characterization showed promising results, though further experiments will be needed to assess the extent and functional properties of the regenerated lenses. The authors then went on to perform a clinical trial in human infants with congenital cataracts, using this surgical strategy in twelve subjects (Lin *et al.*, 2016). They observed healing of the capsular opening within one month, and formation of a transparent biconvex lens structure by three months, that thickened to almost native parameters by eight months post-surgery. A low rate of post-operative complications was also reported. This constituted the first clinical trial for lens regeneration in humans. Longer term follow-up studies will be needed to assess the success of the procedure on visual outcome in these patients. In addition, some preliminary indications from that study suggest an age dependent decrease in human lens regenerative capacity and thus, other forms of induction of regeneration will be possibly required if the strategy were to be applied to older patients. This emphasizes the need of studying alternative animal models such as the newt with unique regenerative abilities that discount aging as a factor.

Finally, stem cell technologies have opened a new breath of possibilities for research as well as translational applications, and the field is benefiting significantly from the insights on the molecular mechanisms of development and regeneration that have been obtained from animal models. For example, knowledge of these mechanisms can be used to better engineer lens organoids from human stem cells, as was recently described by Murphy *et al.,* (2018).

… and onto the future

Overall the accumulated knowledge on vertebrate regeneration, and particularly on lens regeneration, which has been intensively studied for over a century, has challenged our thinking about cell plasticity. Indeed, plasticity is now starting to be recognized more as a normal physiological phenomenon that promotes repair/regeneration after injury, rather than a rarity observed in some obscure organisms. Thus, to the ongoing question of how do differentiated cells gain the capacity for plasticity, we should add the equally important question of how is plasticity prevented or restrained in differentiated tissues. The paradigm of lens regeneration continues to offer an ideal scenario to address these and other fundamental biological questions. The fact that the newt lens rejuvenates every time it regenerates no matter its age, presents a new Pandora's box to uncover. In the era of molecular biology, organoids, gene editing, and "omics", a committed group of regeneration researchers around the world continues to generate new tools that can be applied to regenerative animal models in order to uncover the secrets of regeneration.

Acknowledgments

The authors are forever grateful to Panagiotis A Tsonis (Takis) for the gift of inciting in us a passion for the field of regeneration and specifically for the field of newt lens regeneration. His contributions to the scientific community go beyond his publications and proposed work. He left an inspired generation of scientists to continue pondering on the very questions touched upon in this review and beyond. This work was supported by NIH grant EY027801 to KDRT. We thank Konstantinos Sousounis for critical reading of the manuscript.

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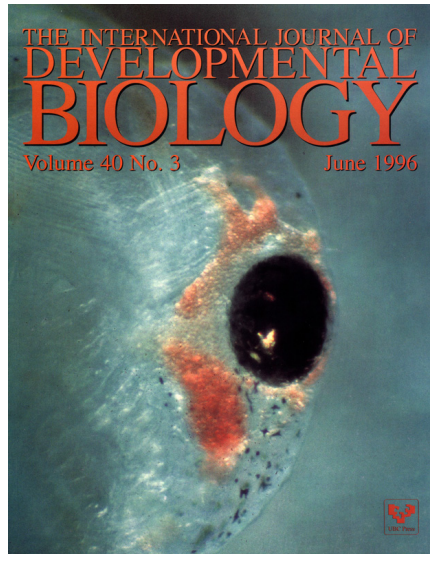

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