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Mechanisms of Cell Regeneration — From Differentiation to Maintenance of Cell Phenotype

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1. Introduction

In adult organisms the regenerative capacity of certain organs or tissues can be limited, resulting in an important clinical challenge for physicians and scientists [1-3].

Regeneration involves the capacity for renewal or recomposition of tissues, organs or even organisms, after considerable physical injury or damage, resulting from pathologies, tumors, congenital diseases or traumas, for example. As a consequence of tissue regeneration, both the composition and the tissue properties are restored, and the newly formed tissue is highly similar to the original tissue. The regenerative capacity is directly related to the presence of stem cells or progenitor cells, which are capable of proliferation and differentiation [4,5]. Tissues that maintain a high proliferative capacity, such as the hematopoietic system, have regenerative capacity even in adult organisms [6].

Cell proliferation occurs in repair processes in general, accompanied by intense production of extracellular matrix, with large amounts of collagen, resulting in the formation of fibrous tissue to occupy the injured area. Although there is lesion filling, both the composition and the tissue properties are different from the original tissue, and the tissue organization pattern is not restored, leading to an altered performance of its functions [2]. Skin healing processes with the presence of scars are examples of tissue repair [3].

Besides the natural processes of regeneration and repair, it is possible, through medical intervention, to fill lesions with natural or synthetic materials, aiming at the recovery of the

compromised area, and conferring certain properties to the tissue, avoiding, for example, exacerbation of the initial lesion or the evolution of degenerative processes [1,7].

The three approaches can be used in tissue engineering, targeting regenerative medicine, as they allow the recovery of compromised areas in different degrees. However, the primary objective is regeneration, recomposition of the original tissue and resumption of the biomechanical and molecular properties, with the normal performance of their functions [1,7-8].

Tissue regeneration involves cell recruitment, growth, proliferation and differentiation, with the latter representing a crucial stage for the success of regeneration, avoiding the formation of fibrous tissue characteristic of the repair [9-12]. Tissues with greater regenerative capacity, such as the skin and liver, intrinsically present cells able to migrate to occupy the affected region, and the same cells maintain the proliferative capacity, enabling occupation of the lesion [3]. In other tissues the regenerative capacity is even more impaired. In the cartilage, for example, the cells remain embedded in the extracellular matrix, and the absence of blood vessels inhibits the presence of other types of component cell in the tissue; even the cell migration and proliferation processes are compromised. In general, regeneration and repair processes do not occur naturally in these cases, requiring surgical intervention to stimulate the subchondral bone marrow, thus enabling the presence of cells capable of tissue repair in the compromised area [13]. Other scientific techniques and methodologies seek alternatives to enable the processes both of repair and of tissue regeneration [9-12,14-16].

Anyhow the final stage of the abovementioned processes, cell differentiation, is critical. An understanding of the mechanisms that lead to the differentiation process in adult organisms allows the proposition of improvements in existing technologies and of alternatives geared towards the optimization of guided tissue regeneration processes, in regenerative medicine.

2. Mechanisms of differentiation

In higher organisms, such as mammals, organs and tissues have limited regeneration capacity. Cell fate determination occurs during the embryonic development of these animals, from the zygote. The zygote, classified as totipotent, has the potential to originate any type of cell, among more than two hundred cell types found in the adult man, for example [12,17]. However, during embryonic development, especially in the gastrulation phase, a more intense and differentiated form of gene expression begins. This phase of embryonic development is still marked by the decrease in cell proliferation capacity and by morphogenetic movement, or cell migration. From this stage on, the morphogens, soluble inductive factors, play a vital role in cell differentiation [17]. The cells gradually exhibit changes in the gene expression pattern, resulting in the development of a specific type of cell, i.e., cell differentiation. The differentiated cells keep the gene expression pattern stable, allowing the expression of its characteristic phenotype [12,14].

The relationship between potentiality and cell differentiation is therefore inversely proportional. With cell differentiation, its potentiality gradually becomes more restricted, and the

more differentiated cells have less capacity to originate other cell types [17-18]. In cell culture systems it is possible to clearly observe situations that favor the proliferative capacity of the cells, such as growth in monolayers with fetal bovine serum supplementation, while generally three-dimensional frameworks, and specific supplementation with growth factors, are inductors for cell differentiation [19-20].

In the adult organism the cells are mostly specialized, or differentiated, form part of complex tissues and organs, and have a low proliferative capacity, limiting the regenerative possibility. It is important to emphasize that some vertebrates maintain more significant regenerative capacities that, in a number of cases, extend to the substitution of complete limbs. Some strategies allow the organism to maintain, albeit in a restricted manner, the tissue regeneration potential, through the: 1) presence of progenitor cells or stem cells; 2) reversal of the cell differentiation process, though limited, by means of dedifferentiation, transdifferentiation or cell reprogramming, the latter with certain restrictions as it is essentially obtained using the artificial manipulation of cells and rarely occurs naturally in the adult organism [8,12].

2.1. Aspects relating to stem cells

The considerable potentiality of embryonic stem cells is lost over the course of the cell differentiation process during the development of the organism. In the adult organism the stem cells, called somatic, maintain the self-renewal capacity but have less potentiality, and are found in some specific tissues, such as the hematopoietic, mesenchymal, nervous, adipose, liver, pancreas and skin tissues as well as others [21-22]. These cells are maintained in the adult organism by two main mechanisms: deterministic and stochastic. In the deterministic model, the division of a stem cell produces another stem cell and a cell with the ability to differentiate, or a progenitor cell. In the stochastic, or random model, some stem cells generate only stem cells, while others generate differentiated cells [6,23-24].

Even in adult organisms the somatic stem cells diminish over time, and older individuals have a smaller amount of somatic stem cells [21,25]. With aging, mechanisms such as cell senescence and apoptosis are apparently related to a decrease in the proliferative capacity of stem cells, resulting from the accumulation of intrinsic (DNA mutations) and extrinsic factors (changes in the specific microenvironment, or niche, in which the stem cells are located) [24,26]. Circumjacent factors (both internal and external), or mediators, are essential for the maintenance of these cells, and can lead to quiescence or activation of the stem cells [24-25,27]. These mediators can be: 1) autocrine or paracrine factors produced by the somatic stem cells, present in the niches; 2) paracrine factors originating from adjacent mesenchymal or stromal cells; 3) molecules present in the ECM, or cell-cell adhesion molecules; 4) factors originating from distant sources, such as blood circulation, immune cells or neurons. The balance between the presence, secretion and expression of these factors modulates cell activity [28].

Some tissues with high cell renewal capacity, such as the hematopoietic tissue, maintain the activity of a population of fast-cycling stem cells so as to maintain the characteristic complexity and their functions. It is proposed that slow-cycling stem cells coexist in these tissues for recruitment in the case of injury and consequent need for tissue regeneration, characterizing inductive and quiescent niches in these tissues. In the case of tissues with a low cell renewal

rate, such as in the brain, muscle and liver, only quiescent slow-cycling stem cells are maintained for activation in the event of injury [28]. In spite of recent advances in the characterization of these niches, not all the modulation factors of somatic stem cells have yet been fully understood [24,28].

2.2. Morphogens and growth factors

Anyhow, it has been established that inductive soluble factors, such as morphogens, are of crucial importance in the cell differentiation process, during embryonic development and in somatic, stem or differentiated cells, since these factors can lead to the abovementioned dedifferentiation and transdifferentiation processes [8]. Morphogens are signaling molecules that induce concentration-dependent cell-specific response. A concentration gradient is formed from the synthesis site of these molecules, and interferes directly in cell response. The initial identification of morphogens in *Xenopus* resulted in the identification of molecules involved in the control of cell growth, belonging to the Fibroblast Growth Factor (FGF) and Transforming Growth Factor Beta (TGF β) families [29-30]. Some examples of morphogens studied are Sonic hedgehog (Shh), in mice and Decapentaplegic (Dpp), in wing development in *Drosophila* [30].

During embryonic development, FGF is responsible for the differentiation of the ventral and posterior mesoderm, while TGF β acts in the epithelial-mesenchymal interactions and BMP (bone morphogenetic protein), a class of TGF β , acts in mesodermal and bone differentiation [12]. In adult organisms the same molecules act as growth factors; for example, FGF acts in the proliferation of chondrocytes [31] while TGF β and BMP act in the differentiation of the cartilaginous and bone tissue, besides other specific functions [32-33]. The cultivation of mesenchymal stem cells in the presence of TGF β allows the reduction of self-renewal markers (Oct4, Stella, Nanos3, and Abcg2), besides inducing the expression of osteoblast dedifferentiation markers (Runx2, Opn, and Col1) [34].

2.3. Inhibition of cyclin-CDK

Since differentiation is inversely proportional to proliferation, both processes occur in a controlled fashion. The high proliferative capacity is maintained in slightly differentiated cells, such as stem cells and progenitor cells. With the differentiation, or specialization process, the proliferative capacity is diminished or even absent, as in tissues with low replacement activity, such as the nervous tissue [35]. Control of the different phases of the cell cycle is a key point both in embryonic development, and in the adult organism. The transition between the G1, S and G2 phases of the interphase, and M phase (mitosis) occurs through a balance of specific signals. Some cells exit the cell cycle, and remain in G0, and in mammals both the G1 sequence, and the escape to G0, occur at the so-called restriction point R, where the cellular metabolic conditions and the gene expression pattern, influenced by extrinsic factors, determine quiescence (G0), growth, differentiation or cell proliferation [36].

The protein p53 is one of those responsible for blocking the cell cycle, especially in the presence of DNA damage. The increase in protein p53 induces the expression of p21 protein, which in

turn binds, through the amino-terminal region, to the cyclin-CDK (cyclin-dependent kinase) complex, usually responsible for cell cycle progression [37-38]. The inhibition of cyclin-CDK enables DNA repair prior to the progression of the cell cycle, since besides the inhibitory action, p21 also binds, through the carboxy-terminal region, to the proliferating cell nuclear antigen (PCNA), blocking DNA replication [39]. The same regulation mechanism can determine the apoptosis process, when there is no repair of the DNA damage. Other proteins that act together with p21, inhibiting CDK, are the proteins p27 and p57 which are both from the KIP (kinase inhibitor protein) family. Besides interfering in cell cycle progression, there is evidence of the action of these proteins in cell differentiation processes [12]. Protein p21 can be expressed independently of p53, and is responsible for the differentiation process in adult organisms and in cell culture systems, being stimulated in the latter by the induction of systems with fetal bovine serum [40].

Another family of inhibitor proteins, INK4 (inhibitors of CDK-4), composed of the proteins p15, p16, p18 and p19, also acts in the control of cell behavior. The cyclin-CDK complex promotes the phosphorylation of the retinoblastoma (pRb) protein, inactivating it and allowing the action of the E2F factor, besides directing the cell to the division process. It may also be related to the progression of tumors in the case of deregulated activity [36]. In the presence of proteins from the INK4 family, this process is inhibited, and the cell cycle does not proceed. However, their performance in cell differentiation is not clearly determined [12,17]. For somatic stem cells, it is suggested that p16 has a repressive action in older organisms, i.e., the reduction of the self-renewal capacity of these cells over time would be due mainly to the regulation of repressive pathways, and not to the loss of permissive capacity for self-renewal, which in spite of reducing the tissue regeneration capacity of these cells, would avoid potentially tumorigenic cell behavior [25].

2.4. Control of gene expression

Besides control of the cell cycle, via inhibition of cyclin-CDK, control of gene expression in non-proliferating cells is an essential cell differentiation control mechanism, maintaining preserved universal patterns in different organisms, both in *Drosophila* and in higher organisms, such as mammals [12, 41].

Certain genes are expressed throughout cell differentiation, and the main point of control of gene expression occurs precisely in DNA transcription. Despite universal gene expression patterns, the greater the complexity of the organism, the more complex the molecular mechanism that acts in this control [42]. Transcription factors are proteins that bind specifically to DNA sequences, inducing or repressing gene expression, and together with chromatin remodeling pattern, influence cell differentiation [42]. The transcription factors act specifically at DNA sites, more specifically the following sequences: promoters, or binding sites for the RNA polymerase; enhancers, binding site for regulatory proteins; and silencers, binding sites for regulatory proteins that block the gene expression [12, 43].

Changes that lead to chromatin remodeling basically occur through the processes of acetylation, methylation and phosphatation of histones, resulting in the removal of positive charges from these proteins, in the case of acetylation and methylation, or adding negative charges to

the phosphate groupings, as a result of phosphorylation. Changes caused by methylation in general hinder gene expression, while acetylation favors access to DNA [44]. In all cases, structural changes modify the access of transcription factors, regulating gene expression, even for cells with the same cell differentiation pattern, i.e., the same genotype can correspond to different phenotypes, due to reversible changes in gene expression, without any changes in the gene content itself [42,45-47].

Control of cell phenotype can be extended beyond gene expression, in post-transcriptional controls, by modulation of the mRNA transcription process, and in post-translational controls, with changes in the proteins for their biological activation. The protein phosphorylation process is one of the most common post-translational changes, and results in the formation, for example, of signal transduction mediators [12,35].

2.5. Influence of the extracellular matrix and cell-cell interaction

The influence of the extracellular matrix (ECM) in the differentiation process involves the presence of factors immobilized in the ECM, such as morphogens, secreted growth factors and chemokine gradient. The ECM can be defined as an interconnected network of macromolecules composed of adhesive proteins (such as fibronectin, vitronectin, tenascin and laminin), structural fibrous proteins (collagen and elastin), glycoproteins and specialized proteins (such as growth factors) [35,48]. The modulation of behaviors such as adhesion, growth, migration, proliferation and differentiation, occurs as a result of the direct influence of the contact of cells with the ECM, or with adjacent cells [6-7,28,48]. The bond with the ECM elements occurs via membrane receptors and results in cytoskeletal reorganization, which in turn triggers a cascade of intracellular events through signal transduction [35,48].

An example of differentiation control is the interaction of cells with fibronectin. Fibronectin is a multiadhesive protein of the ECM. At least 20 isoforms of this protein obtained through the alternative processing of the transcript of a single gene are known. They are proteins that act both in migration and in cell differentiation, during embryogenesis and in adult organisms [35]. The intracellular events of binding with fibronectin are a result of their action with transmembrane receptors, the integrins. Integrins are dimeric glycoproteins, formed by pairs of different combinations of the subunits α and β [48]. In the organization of epithelia, such as of the skin, the basal layers are kept in contact with the ECM through the bond with fibronectin. As the cells migrate to the upper stratum, this bond is lost and cell differentiation occurs [12]. In the keratinocytes, for example, the differential expression of receptors for fibronectin was observed throughout the cell differentiation process. These receptors are the integrins: $\alpha 5 \beta 1$, which recognize RGD sequences of fibronectin and act in cell growth and initial migration; $\alpha v \beta 6$, not characteristic of cells from the basal layer. It can activate the growth factor TGF β , directing differentiation, and is present mainly in the tissue repair process; and $\alpha v \beta 1$, which is a low-affinity receptor that facilitates the final stages of cell migration. The alternation in the expression of the integrins allows the migration and differentiation of these cells [48]. Similarly, in the pulmonary epithelium the tissue response to pathological condition of pulmonary fibrosis to integrin $\alpha v \beta 6$ is responsible for the activation of the constituent expression of TGF β , acting as epithelial transdifferentiation regulators during fibrogenesis [49-50].

Not only the fibronectins, but also the proteoglycans, act in tissue organization and direct differentiation. These glycoproteins have repetitive chains of disaccharides that are generally acid, bonded to a protein nucleus. Besides the structural function and the high hydrophilicity of this molecule, these molecules have the ability to bind to other diffuse proteins in the ECM, acting direct or indirectly in cell differentiation. Betaglycan and decorin bind to TGF β , and heparan sulfate to several other morphogens, such as FGF and Wnt [12]. There have been reports of the non-differentiation of mice embryo stem cells due to the reduction of heparan sulfate sulphonation, impeding the response to FGF [51].

Collagen is the most abundant component in the ECM, and is directly linked to cell differentiation processes. This structural fibrous protein begins to be secreted during embryonic development, in the gastrulation stage, concomitant to differentiation of the three germ layers [52]. In the adult organism the action of this protein in maintaining differentiation and during the tissue repair and regeneration processes has been described for different cells such as fibroblasts, hepatocytes, pancreatic acinar, thyroid epithelial, mammary epithelial and others [12]. Mammary epithelial cells of mice exhibit cuboid dispersed morphology, when cultivated on surfaces with type I collagen, preventing their differentiation and functional activation in the production of β -casein [48]. Similarly, hepatocyte cultures also tend to firmly adhere to surfaces coated with a fine film of type I collagen, are polygonal, dispersed and with suppression of specific function [48]. However, three-dimensional collagen gels induce the cells to cytoskeletal reorganization and the resumption of differential gene expression, leading to the specialized phenotype of the cells. Mammary epithelial cells in collagen gels can form duct-like structures and secrete milk proteins, such as β -casein. Hepatocytes cultivated in three-dimensional collagen gels present albumin secretion [12,48].

Studies reporting the *in vitro* differentiation of chondrocytes are also demonstrative of the action of the ECM elements in this process. Chondrocytes cultivated in two-dimensional monolayers appear dedifferentiated, with flat morphology and expressing mainly type I collagen, characteristic of fibrocartilage, or fibrous tissues. With the maintenance of three-dimensional culture systems, the chondrocytes resume the expression pattern characteristic of type II collagen, and synthesis of proteoglycans [53-55]. The binding of chondrocytes to the ECM allows the modulation and maintenance of the differentiated phenotype of the cells, being one of the principles of the chondrocyte implantation technique, a tissue engineering method applied to chondral regeneration. This binding occurs via integrin, collagen receptors and laminins, which in turn signal the modulation of cell behavior via Wnt, nitric oxide, retinoic acid and protein kinase C [55]. Most integrins expressed in chondrocytes present β 1 chain, and its absence brings about important changes in the cartilage phenotype, being one of the factors of apoptosis, together with integrin $\alpha\beta$ 5 [55].

ECM degradation also directly influences cell differentiation, from embryonic morphogenesis to adult tissues. The proteolytic cleavage of the ECM elements, through the action of metalloproteinases, serine proteases and cysteine proteases, and the consequent solubilization of their components, signal cell behaviors. Morphogens and growth factors such as TGF β which are embedded in the ECM, often bound to proteoglycans, are made available, signaling the tissue repair or regeneration process. During salivary gland formation the development of ramifi-

cations occurs in the presence of collagenase inhibitors, favoring the interaction of the cells with the ECM [12]. Mammary gland involution results in the cleavage of laminin, whose fragments bind to EGF receptors, resulting in increased cell migration, which together with collagen cleavage and release of soluble factors such as Wnt, TGF β and FGF, favor tissue remodeling [12].

Tissue remodeling in bone formation, starting from endochondral ossification, is another example of differentiation regulation by the specific degradation of the ECM. The substitution of chondrocytes in the tissue maturation process involves the presence of a lecithin, galectin-3, located in the ECM and with antimitotic action. During the remodeling of the ECM, this lecithin is cleaved and inactivated, allowing a sequence of events that leads to ossification. In cases where this lecithin is not degraded, chondrocyte apoptosis does not occur, resulting in bone formation defects [56-57].

Besides the interaction directly with ECM elements, cell-cell interaction also directs the differentiation process, but expressively in embryonic development and epithelial tissues. Among the Cell Adhesion Molecules (CAMs), the cadherins, a superfamily of calcium-dependent transmembrane proteins, are important cell differentiation mediators. N-cadherin is responsible for cell migration, both in embryonic development and in adult tissues, while E-cadherin is expressed mainly in the embryonic and epithelial tissues [35]. Returning to the example of mammary epithelial cells, anti-integrin β 1 antibodies resulted in the blocking of β -casein synthesis, while anti-E-cadherin antibodies do not interfere in protein synthesis, demonstrating the influence of ECM in the transduction of signals independent of cell-cell interaction [48].

Therefore cell differentiation mechanisms are obtained by complex relations between intrinsic factors and extrinsic influences, which trigger signaling reactions and modulate cell behavior as well as gene expression. In general the factors that act in cell differentiation from receptors on the cell surface lead to transduction of signals via protein kinase, activating intracellular phosphorylation cascades and culminating in gene expression regulation, besides cytoskeletal reorganization [4,12,35].

It is important to stress that the factors responsible for cell behavior modulation during differentiation, do not only apply to somatic stem cells. Other types of cell can be involved in dedifferentiation and transdifferentiation processes. The use of animal models is essential in establishing the concepts presented and the understanding of cell differentiation mechanisms, as described below.

3. Regeneration in animal models

Given the importance of differentiation for the acquisition of cell functions concerning the formation and maintenance of the organism, it is advisable to gather information on the regeneration process that, as mentioned previously, involves the capacity for recomposition and renewal of parts through the remodeling of somatic tissue [10,14]. Regeneration in an adult

animal appears to be a noteworthy example of postembryonic morphogenesis. It involves recognition of tissue loss or injury, followed by mechanisms of reconstruction or recovery of the structure in question [58].

One of the mechanisms associated with natural regeneration is **dedifferentiation**, which involves a terminally differentiated cell returning to a less differentiated phase of its own lineage. This process allows the cell to proliferate again before redifferentiation, which leads to the substitution of these cells that were lost [59]. Cell dedifferentiation changes a program that directs the specific function of a somatic cell to another program or to proliferation, regardless of whether the destination is the same as the origin [60].

Transdifferentiation is another natural mechanism that was observed for the first time in the regeneration of the salamander lens over 100 years ago. As mentioned above, this process involves the conversion of a differentiated cell type into another [12]. This occurs because the cells recede to a point where their change of lineage becomes possible [59].

We should designate another process, which aims to induce differentiated cells to revert their pluripotency: **reprogramming**. From this point on, they can differentiate into almost any type of cell. Although reprogramming occurs naturally during fertilization to produce totipotent cells that can differentiate into any type of cell, it has not yet been formally shown as a true regenerative response. Moreover, reprogramming avoids the need to use embryos for regenerative therapies using differentiated cells created from a patient. From the clinical point of view, this has the additional bonus of circumventing the immunological problems associated with grafting (such as transplant rejection and graft versus host diseases) [59].

3.1. Regeneration in different animals

Some animals, such as starfish, planaria (flatworms) and *Hydra* have significant regenerative capacity, and can originate complete specimens from small fragments [10,14]. It is interesting to see the comparison between regeneration and fragmentation, fission and budding, which are forms of propagation in invertebrates. Fragmentation is the simplest form of asexual reproduction and is essentially identical to regeneration after cross sectioning. Fragmentation can occur in animals that can be broken by external forces and that regenerate completely from each lost part. Fission, in contrast to fragmentation, is generally an endogenous process [58,61]. Returning to the topic of regeneration, we present below some model systems:

- **Zebrafish:** Fins and tail of zebrafish can regenerate after amputation. The phenomena called appendage regeneration have been studied at length. This fish can also regenerate cardiac tissue after resection or the destruction of cardiomyocytes. In the regeneration of the zebrafish fin after amputation, there are four stages called "epithelialization, regeneration or healing of wounds", "blastema formation", "regenerative consequence" and "termination". In epithelialization, the proximal epidermis migrates to cover the stump and to form a 3-4-cell thick layer. Inflammation then proceeds to clean the clotted plasma and cell debris. For blastema formation, there is histolysis and remodeling of the extracellular matrix; cells to be released by histolysis start to dedifferentiate in this period. Then blastema aggregation is achieved through an accentuated increase in the mitosis, which is dependent on factors

from the wound and regeneration of the epidermis and nerves. A notable feature is the rare presence of apoptotic cells in the blastema despite the presence of avascular tissue, which could present hypoxia and potentially be susceptible to apoptosis.

- **Xenopus:** Fins and tail of *Xenopus* larvae can regenerate after amputation.
- **Hydra:** When cross sectioned, *Hydra* will regenerate a head (from the lower piece resulting from the sectioning) or a foot (from the upper piece), which will always depend on the relative position of the regenerated structure in the piece being regenerated. In other words, the sectioning surface closest to the head forms a head. The same applies to the foot, which shows well defined general polarity. Regeneration in *Hydra*, starting from a small fragment of the spine, does not involve an initial increase in size. This results in a small animal, which will have its normal size reestablished with feeding [10].
- **Urodeles:** Salamanders and tritons (urodeles) have major regenerative capacity. Actually, so far as we known, the Urodeles are the only adult vertebrates able to regenerate their limbs. The regenerative capacity of the adult triton covers different regions, such as maxilla, crystalline lens, retina, large sections of the heart, and its limbs and tails, in response to tissue damage or even amputation. The salamander can regenerate its limbs and tail, ocular tissues, the intestine, and small sections of the heart. Limb regeneration also takes place in salamanders, where amputated limbs are covered by the epidermis, and the immature cells accumulate and proliferate below them, forming the "blastema". The blastema, which is coated by the newly formed epidermis, involves undifferentiated cells that are restricted to the musculoskeletal cells, chondrocytes, Schwann cells, and mesenchymal fibroblasts. Heart tissue can also regenerate after resectioning or the destruction of cardiomyocytes.

There is a large gap between the examples presented previously and the regenerative capacity in other vertebrates. With the exception of Urodeles, regeneration in vertebrates is classified as very limited. In mammals, it is much more restricted, being limited to regeneration of the liver, when part of this is removed, or even by a bone fracture healing process. The regeneration of lost limbs is not possible [10,14]. Cardiomyogenesis was observed in murine hearts only at less than one week of age. The regeneration of vertebrates implies dedifferentiation. However, the factors distinguishing vertebrates with or without regenerative capacity still need to be clarified [60].

An interesting view is that regeneration is an essential attribute of metazoa, and was secondarily lost in closely related species or more distant groups [58]. In analyzing the mechanisms that form the basis of regenerative responses in Urodeles, a comparative study with mammals was allowed. Thus it is possible to identify the primary differences between Urodeles and mammals, especially with regards to the evolutionary bases for regeneration [58]. Nevertheless, the reasons that lead to the understanding of the animals having such a different regenerative capacity are not clear. Several hypotheses have been raised to this effect [58], which illustrates the complexity of the problem: (1) some species have a fixed number of segments in adults, and a very large number of non-regenerative species share this characteristic; (2) if amputation removes a structure that is not critical for survival, then the regenerative capacity could be lost, as it would not be a selective advantage; (3) if some species have low rates of

amputation of their segments, this could lead to a loss of regenerative capacity; (4) if fission is present in a given species, this may diminish the selective advantages for regeneration.

On the other hand, some observations indicate that these regeneration responses have not been totally lost in these species. This idea is supported by the fact that many species have good regeneration capacity as embryos (including humans), but this capacity is gradually lost (or silenced) during development and aging [62]. Many aspects of this matter still need to be discussed.

3.2. Response to injury

The regenerative response is initiated through recognition of the loss of tissue or local wound. It is not yet understood which factors initiate this response, as there are potentially lots of signals involved. Nowadays, events such as *bioelectric signaling*, *thrombin activation* and its hemostasis, the possible *influence of the immune response* in regeneration and the formation of a *wound epithelium* are considered relevant.

- **Bioelectric signaling** involves electric currents in lesion that flow after amputation of limbs and reflects the geometry of the altered tissue and consequently, the electrical resistance. The amputation of a salamander limb produces derivation of low resistance at the end of the stump, through which the ionic current flows during the first days [63]. Wounds are generally electrically positive in relation to the more proximal uninjured areas. Recent experiences in the caudal regeneration of *Xenopus* larvae indicate the existence of important activity of the V-ATPase proton pump [64-65]. The inhibition of this pump blocked regeneration, while maintenance of the V-ATPase expression maintained the regenerative capacity. The need for the presence of nerves for effective regeneration in limbs has already been described. It was proposed that the need to provide a nerve for regeneration is the aspect influenced by circuits of internal currents produced by this nerve [66]. Another important point in regeneration in planaria, in which the regeneration polarity can be manipulated by applied electrical fields, so that the animal heads are always formed when facing the cathode [67].
- **Thrombin activation** is an essential regulator of the response to injury in vertebrates and has become a strong candidate to initiate the regenerative response in salamanders as observed in the repair of the intestine [68], limbs [69] and heart [70]. Clots are formed as a result of the action of thrombin protease on the plasma fibrinogen, and thrombin activation from prothrombin can be regulated by hemostasis. The possibility that local thrombin activation could be a regeneration signal originated from studies in which salamander myotubes cultivated *in vitro* returned to the cell cycle through thrombin-linked activity [71]. After the removal of the crystalline lens in tritons, thrombin activity appears transitorily in the dorsal pupillary margin of the iris and can be blocked by the introduction of inhibitors in the ocular chamber [72]. This intervention reduces cell cycle reentry by the pigment epithelial cells (PECs) on the dorsal margin and inhibits crystalline lens regeneration [73]. In the same model, cell proliferation is dependent on the activity of the fibroblast growth factor 2 (FGF2), while activation of the Wnt signaling system determines the crystalline lens

regeneration site [74]. During regeneration of the liver in mammals, some evidence suggests that, after hepatectomy, the release of serotonin by the platelets is a fundamental signal for the onset of hepatocyte proliferation [75]. Platelet activation is another aspect of the thrombin-dependent response to injury.

- **Formation of the scar epithelium on wounds** is an early response to the injury and consists of the migration of epithelial cells to the amputation plane or tissue lesion [67]. The wound epithelium assumes a specialized identity and plays an important role in subsequent regeneration events. In some cases, the formation of epithelium on the wound does not occur. In crystalline lens regeneration, epithelial transdifferentiation occurs at the site without involving the formation of a wound epithelium. In cardiac regeneration in zebrafish there is early and generalized activation of the epicardium, which can perform a role similar to that of the wound epithelium [76]. The role of the wound epithelium is not yet fully understood, but some points can be raised. The formation of wound epithelium in salamanders can be avoided by suturing skin over the extremity of a limb or amputated tail. This procedure allows the wound to heal, but prevents limb regeneration. The wound epithelium can provide a distal limit to standardize mechanisms during regeneration, even though positional identity is usually considered a function of the mesenchymal cells [77]. It has been suggested that the epidermal cells of different circumferential identities can migrate and form a functional wound epithelium [78]. The formation of this wound epithelium is a target for a variety of regulatory events. The formation of the wound epithelium has been shown to be related to FGF20a activity in zebrafish. This can be regulated by Wnt 10a and Wnt5b [79], where it was shown that inhibition of signaling via Wnt/ β -catenin leads to a decrease in FGF20a expression.

Following removal of the apical region of the triton ventricle, the heart seals, by contraction, around the clot. Adult cardiomyocytes re-enter the cell cycle and the division of a peripheral zone of the clot. If the animal is injected with tritiated thymidine to identify the cells that are in S phase, about 10% of the cardiomyocytes in this region are marked in the period of a day. In experimental comparisons with the heart of an adult mammal, very few cells are marked after injury [22,80]. In tritons, after crystalline lens removal, the population of regenerative cells involves pigment epithelial cells, which are invariably located on the dorsal pupillary margin of the iris. These cells re-enter the cell cycle, lose their pigment granules and are converted into crystalline lens cells, a process known as transdifferentiation [22,80].

After the amputation or tissue injury, there is the regeneration and supply of the nerve to the damaged region. Normal limbs require the presence of nerves to regenerate. Limbs that have had nerves removed prior to amputation do not regenerate. However, aneurogenic limbs can regenerate normally [10]. In most cases, this regeneration involves only the axonal extension, so as to reestablish functional contacts with the newly formed tissue. In the case of tail regeneration in salamanders, or head regeneration in *Hydra*, the generation of new nervous cells can also be involved. It has been observed that regeneration is dependent on and concomitant to the nerve supply [67]. This dependence is widely conserved phylogenetically, being observed not only in several contexts in vertebrates, but also in examples of echinoderms [81] and annelid regeneration [82]. This dependence shows the clearest example of the

difference between development and regeneration. The dependence on nerve regeneration may be a means of ensuring that the regenerated tissue is functional.

The **plasticity** of cell differentiation-capacity to differentiate into a cell range featuring distinct functionalities-provides a convenient cell assay for the comparison of a differentiated cell in Urodeles with its congener in mammals. The plasticity of differentiated cells is a remarkable characteristic in heart regeneration in salamanders, as it depends on the capacity of the cardiomyocytes close to the lesion to re-enter the cell cycle. Cardiomyocytes of adult salamander ventricles when dissociated in culture re-enter the S phase. About one third of these cells progress through mitosis and undergo successive cell divisions, in contrast to their counterparts in mammals. This is accomplished without major loss of differentiated properties and cells resume their heartbeats after cytokinesis [83].

It is important to recognize that there are examples of regeneration in mammals that involve plasticity. An example in a mammal that depends on the plasticity of the differentiated cells is in the liver that appears to be comparable to cardiac regeneration in salamanders, as the hepatocytes split without loss of differentiated function [84]. The regeneration of the myelinated peripheral nerves requires the reentry of Schwann cells in the cell cycle, with the loss of their differentiated properties, such as myelin expression, and the acquisition of a phenotype that facilitates axon regeneration, before redifferentiating together with the regeneration axon [83].

In appendages (limb and tail) of urodeles under regeneration, multinucleated myotubes or striated myofibers undergo cellularization to produce mononucleated cells capable of division. The nuclei of multinucleated muscle cells can also reenter in the S phase, although this is not apparently necessary for cellularization to occur. Thus multinucleated skeletal myotubes are formed by the fusion of mononucleated precursor cells. The myotube enters a post-mitotic imprisonment state in which it is totally refractory to the growth factors that stimulate the division of its precursors. The change in the cell, from mononucleated to multinucleated, together with the stationary phase after mitosis, provides two indices for the reversal of the myogenic phenotype (dedifferentiation) [22,80]. In tritons, after limb amputation, epidermal cells migrate to the surface of the wound, which is extremely important for subsequent growth. A blastema forms from cells beneath the wound epidermis, which lose their differentiated nature and begin to divide, thus producing a regenerated limb. As the limb regenerates, these cells differentiate into cartilage, muscles and connective tissue. The question regarding this phenomenon is whether the cells that differentiate into cartilage and muscles in the blastema remain faithful to their original types, i.e., whether previously muscle cells are, necessarily, those that will produce the muscle cells after regeneration.

In vitro, triton myotubes clearly differ from their vertebrate counterparts, in which these enter and cross the S phase after stimulation with fetal serum in the culture. The nuclei in the myotubes duplicate their DNA content and are detained stably in G2. The response to the serum is not observed for other myotubes of vertebrates, with the exception of rat cells in which both copies of the retinoblastoma gene are missing. pRb plays a familiar and essential role in regulating the G1-S phase transition. Several lines of evidence indicate that this is crucially important to maintain the differentiation state in myotubes of vertebrates, not only for the

stable imprisonment of the cell cycle, but also for transcription starting from certain muscle promoters that depend on the activation of limbs of the *myocyte enhancer factor 2* (MEF2), a family of transcription factors. Triton myotubes express pRb, but the serum stimulates a pathway that leads to its inactivation by phosphorylation, and consequently, causes progression from G1 to S phase.

Serum is the soluble fraction of clotted blood, and results from prothrombin activation to generate the serine protease thrombin. Thrombin activates the coagulation cascade and other events to mediate the response to the injury. When crude prothrombin is activated *in vitro*, the resulting thrombin preparations contain a distinct activity, which acts directly on the triton myotubes in a serum-free medium [80]. Vascular prothrombin activation following injury occurs in relation to a protease complex known as *tissue factor*, which is mounted on the cell surface. Thrombin formation is subject to strict spatial and temporal regulation, as it is essential for clot formation to be restricted to the area of the wound, and not to spread. It is speculated that regeneration in Urodeles-in the heart, limb, or even eye-is linked to acute events of injuries or the removal of tissue from the thrombin activity site. Thrombin activity is locally increased in the early mesenchymal blastema of the limb, and prothrombin has been seen to be selectively activated over the dorsal margin of the iris after injury in recent discoveries [80].

A critical contribution to tissue standardization can also come from the fibroblasts of the connective tissue of the dermis, and the degree of alteration of its differentiated state is not yet clear [85].

3.3. Morphallaxis, epimorphosis and tissue regeneration

Typically, the regeneration process can be distinguished in two types: *Morphallaxis*-Regeneration occurs through the re-standardization and delimitation of existing tissue, with little growth of new tissues-and *Epimorphosis*-where there is the growth of correctly standardized new structures[10,14]. There has also been talk of a third type, called *tissue regeneration*.

Morphallaxis [*morph* (form)+*allaxis* (change/substitution)] involves remodeling of the without proliferation. The absence of cell division is not currently regarded as an essential criterion to define morphallaxis. The important criterion is the extreme remodeling of the remaining structures, as can be seen in the hydrozoans, planarian <??>, some annelids and other invertebrate animals. In *Hydra* regeneration occurs dynamically, with stable and continuous cell proliferation. As the tissues grow, the cells shift along the body column. The adult *Hydra* needs to lose cells continually to maintain its size. The loss of cells occurs at the ends of the tentacles and in the basal disc of the budding foot, and most of the excessive production of cells is used in the asexual budding of new *Hydra* from the body column [10]. Such dynamic re-standardization of the cells occurs through mechanisms that confer regenerative capacity to this animal.

Hydra has two organizing regions (one at each end, namely, hypostome and basal disc) which confer its general polarity. There is also interaction between the head inhibition gradient – which prevents the formation of other heads in other regions-and a positional value gradient – which determines the threshold at which head inhibition occurs-in the regeneration process. Such organization and interaction confer a dimensional arrangement that precludes (or

hinders) malformations, or even incoherence between their positioning. It is assumed that the head inhibitor is a secreted factor, produced by the head itself, which spreads downwards along the body column and is degraded at the basal extremity. It is also assumed that the positional value gradient is an intrinsic property of the cells. Both are linear with values at a constant rate of decrease as they move further away from the head. Accordingly, the specification of a region of the head on the surface of the section is the first basic stage in this morphallactic regeneration when the head region is removed [10].

As regards **Epimorphosis** [*epi* (on/over)+*morph* (form)] it is defined today as the method of regeneration, where a blastema, a mass of proliferative cells located at the tip of the amputation stump, is formed [67,86]. As pointed out previously, there is standardized regeneration of new structures which leads us to reflect on the fact that cells completely differentiated from the mature limb return to the cell cycle, dedifferentiating to redifferentiate in the regeneration process, forming different types of cell, with restructuring of the injured limb [10].

Epimorphic regeneration occurs in Urodele amphibians. As a problem in cellular and molecular biology, regeneration in Urodeles provides information of considerable importance about the reversal and the plasticity of the differentiated state of cells. Limb regeneration thus adds up to a system in which the key for studying the positional identity in cells is established. Notwithstanding the usefulness of studies about development, evolution and phylogeny, it is a widespread concern of regeneration research to understand the material differences between species that regenerate and those that do not. In salamanders, although the tissue restoration process occurs differently in the heart and in the limbs, the result appears to depend on the plasticity of the differentiated cells that remain after tissue removal [22,86]. Salamanders can regenerate an entire limb from a blastema. In mammals, digit-tip regeneration does not originate from a blastema, but instead from progenitor cells in the ungual bed [87]. The blastema of a limb consists of a group of mesenchymal stem cells at the end of the stump.

Regeneration always occurs distally in relation to the sectioned surface, which allows the replacement of the lost part of the limb. The development of the blastema, as well as the nature of the structures that it originates, depend on the amputation site and not on the nature of the nearest tissues. The limb undergoing regeneration identifies the positional value at the amputation site, regenerating all the positional values distal to this site. Note that epimorphic regeneration involves the retention of embryonic processes, such as the ability to specify new positional values, which are encoded as a property in gradient, partly on the cell surface, and whose relevant behavior for axial specification (growth, movement and adhesion) is a function of the expression of this property in relation to the neighboring cells [10].

The strategy used by Urodeles in regenerating most structures is, therefore, the re-specification of differentiated cells into local progenitor cells, rather than the activation of a pluripotent cell. This is called dedifferentiation. Thus if the epithelial cells of the iris are transplanted to the blastema of the limb, they produce a crystalline lens, and blastemas of limbs always produce a limb after transplantation, even after transfer to the anterior chamber of the eye. This contrasts with the recent results of considerable plasticity of stem cells following transplantation in mammals. At present there is no evidence that adult stem cells can contribute to regeneration in limbs of Urodeles, although it is not possible to completely rule out this possibility. An

advantage of the regeneration mechanism in Urodeles may be that it allows the progenitor cells to derive local signals from their differentiated parental cells [22,86].

Tissue regeneration is another type of reparative regeneration that involves proliferation, close to the cut surface, but unlike epimorphosis, there is no formation of a blastema [67,86]. It involves the restoration of tissues damaged by the removal of dead cells, the proliferation of progenitor cells and of functional restoration of the tissues [67]. Classical examples of this type of regeneration in mammals are the skin, bones and regeneration of the skeletal muscle, although the degree of regeneration in mammals is very limited. Liver regeneration, in turn, involves enlargement of the remaining lobules by proliferation of cells throughout the organ to make up for the lost mass, without this resulting in the restoration of the initial morphology. Once removed the lobules do not grow back [88].

It is possible for regeneration to occur in the same animal through more than one model. Thus the limits between these definitions are often not particularly clear.

3.4. Positional identity and polarity in regeneration

We now know that this positional identity is encoded in the cell membrane by a PD gradient of glycoproteins from the cell surface [89].

An important issue in regeneration is how the blastema cells identify their spatial position. Blastema cells are derived by dedifferentiation of adult mesenchymal cells at the amputation plane. If a blastema is removed from its limb by transection to the amputation plane and is transplanted to an adequate site, such as the anterior chamber of the eye or of a lesion in the connective tissue of the dorsal fin, they form a normal regenerated appendage/tissue. The cells derived from the blastema after amputation, at any level on the proximodistal axis, will produce the distal structures. This property is called positional memory. Blastema cells are derived by dedifferentiation from adult mesenchymal cells at the amputation plane, and they derive critical suggestions about their identity and potentiality of their precursors. When the regenerative cells are transplanted in tissues in the salamander, they keep their original identity. Position memory is a critical aspect for limb regeneration autonomy, because it specifies the initial population of blastema cells in relation to the extension of the axis to be regenerated. An understanding of its molecular basis is generally important for our appreciation of how stem cells are specified to produce different structures, instead of different cell types [83].

It is accepted that gradients of morphogens associated with cell-cell interactions provide the blastema cells with information on their three-dimensional position, which is similar to what happens during morphogenesis [71]. However, unlike development, the blastema cells need to know their proximodistal level to allow regeneration to occur from the correct portion. Interaction studies between proximodistal portions of the limbs in salamander blastemas have suggested that cell adhesion, movement and division can be important in the positional identity expressed in cells [89]. The hypothesis was that the proximodistal identity is encoded by a molecule or molecules to the cell surface, possibly as a level of gradual expression along the proximodistal axis. These considerations led to the identification of *Prod 1*. *Prod 1* encodes

a small protein that is bound to the cell surface by a glycosylphosphatidylinositol (GPI) anchor, is apparently analogous to the mammalian CD59 protein and is implicated in positional identity determination. The mammalian CD59 protein is associated with inhibition of the terminal phase of activation of the complement system, and it is also able to mediate the activation of intracellular tyrosine kinases. The distal cells of the blastema of axolotl larvae are converted experimentally into proximal cells when expressing *Prod 1* [5,90]. The *Nag* protein was also identified as a binder to *Prod 1*. *Nag* acts as a mitogenic factor for blastema cells [90].

The head formation process, which occurs during the regeneration of *Hydra*, involves apoptosis, proliferation, and re-specification of cells at the amputation plane [91]. This leads to the formation of a transitory organizer in the transient head, which apparently involves the signaling pathways wnt and β -catenin. There are data showing that regeneration in planaria also occurs with the participation of β -catenin, as mentioned above.

3.5. Mobilization of regenerative cells

The determination of the origin of blastema cells has been one of the main concerns of regenerative biology for a long time. Theoretically, the blastema cells could originate from: (1) mature cells dedifferentiated from the stump of the limb, (2) the stem cells or reserve cells of the stump, (3) the plasticity of mature cells of the stump and (4) progenitor cells from the rest of the body. Transplantation experiments using cells marked with green fluorescent protein (GFP) of several tissues of the axolotl showed that grafted cells dedifferentiate, proliferate, and redifferentiate into cells restricted to their lineage of origin (5). The tracking of osteoblasts in the regeneration of zebrafish fins demonstrated the dedifferentiation of osteoblasts and their subsequent redifferentiation [92]. However, it is possible that the resident stem cells are involved in appendage regeneration [5], especially in the case of the skeletal muscle, which are accompanied by a population of stem cells called satellite cells [93].

All the tissues from the distal stump of the limb appear to contribute somehow to the formation of the blastema, except the epidermis. The dedifferentiation of mature cells is believed to be an important mechanism for blastema formation [86]. Reports have confirmed that dedifferentiated cells acquire proliferative and migrant capacities, necessary for the formation of the blastema [59,90]. There is a dichotomy between a reserve of progenitor cells in relation to the local plasticity of the differentiated cells. Previous studies suggested that the regeneration of the zebrafish myocardium occurs through reactivation of the cell cycle of adult cardiomyocytes [94]; more recently, other data has indicated that the new myocardium originates from progenitor cells [76].

Besides dedifferentiation, there is evidence of the contribution of adult stem cells to the blastema [95-96]. It is believed that Urodeles, and perhaps some fish, may contain a different muscle progenitor cell, which originates in the muscle fibers that dissociate through the dedifferentiation process. The multinucleated muscle fibers fragment into mononuclear cells that recover their proliferative capacity and migrate to the blastema. These progenitor cells presumably produce a new muscle together with the activated satellite cells[5].

In vitro, skeletal salamander myotubes are able to re-enter the S phase in response to a thrombin-derived binder present in the serum [71]. Myotubes of mammals and other vertebrates do not respond to stimulation with serum; hence there is a distinct property of differentiated cells in the salamander. A second aspect of the plasticity of skeletal muscle in salamander is the conversion of multinucleated myotubes and myofibers into viable mononuclear cells. In tritons, muscle satellite cells, during regeneration of the limbs, express *Pax-7* [97] when activated. In this model, there appears to be a balance between the satellite cells and cellularization originating from myogenic cells. A recent study of mandibular regeneration in tritons suggested that both sources are important [98]. On the other hand, in the regeneration of the tail of *Xenopus* tadpoles, regeneration appears to occur exclusively from satellite cells [95]. Triton myotubes can be prevented from reentering the cell cycle both by X-radiation and by p16INK4. Such myotubes, when marked and implanted into a blastema of a limb under regeneration, were effectively converted to the mononucleated cells. Hence although reentry and cellularization occur at the same time, after implantation, they are not mechanically linked [80].

An important impetus for studying cellularization came from the recent discovery of two methods that induce this process in rat myotubes. In an approach, the mononuclear cells were transfected with the homeobox gene *Msx-1*. Several studies have previously indicated that *Msx* genes promote cell proliferation, and that their expression is inversely correlated with differentiation. After the fusion of the transfected cells, the expression of *Msx-1* was induced in myotubes and this led to a decrease in the expression of myogenic regulatory genes. About 5% of myotubes were induced to cleave into viable fragments and another 5% fragmented into mononuclear cells, which proliferated. In some cases, the clonal progeny of a single myotube was isolated, propagated and showed itself capable of chondrogenic, adipogenic or myogenic differentiation, depending on the culture conditions. It has been proposed that *Msx-1* is a master regulator of the program for cellularization that is expressed in regeneration in Urodeles, and that can also induce this program in mammal myotubes [80].

A second impetus for the analysis of these matters comes from the application of “chemical genetics”. A large combinatorial library of tritiated and substituted purines was exhibited to identify a compound that induces mammalian myotubes to regenerate. A compound, with substituents methoxybenzyl and isopropyl, effectively fragments the myotubes over 24 hours to produce viable mononuclear cells that can divide and also fuse together once more for myotubes to reform. This compound, called *myoseverin*, appears to have two activities in mammalian myotubes. Firstly, it depolymerizes microtubules. Secondly, it induces changes in the expression of a specific set of genes that are involved in repair, wound healing and regeneration. Although there are other agents that can fragment microtubules, they do not tend to produce viable mononuclear cells that can fuse into myotubes again. Therefore, it is possible that both activities, i.e., depolymerization of microtubules and changes in the gene expression, are important for the generation of viable mononuclear cells [80].

A possibility is that the compound responsible for the fragmentation of myotubes can also regulate genes that are related in tissue remodeling and repair. The alternative and most appealing hypothesis is that *myoseverin* can activate the expression of a program that acts as

mediator of cellularization and other functions that are important for regeneration. DNA microarray analysis has identified approximately 90 genes so far. Many of these genes belong to categories that are regulated in fibroblasts in response to the serum [80].

The results with myoseverin raised the issue of the identity of the endogenous signal that activates this response in the muscle fibers of Urodeles during the initial phase of regeneration. A recent study presented evidence proving that binders present in extracts from the start of the regeneration in triton limbs can induce the myotubes of tritons and of rats to submit to cell cycle reentry and cellularization. Cellularization indicates the presence of myoseverin as an activity both in triton cells and in mouse cells. However, reentry in S phase of rat myotubes is surprising in view of the previously discussed evidence pointing to a clear difference between the capacity for response to serum and thrombin between triton and rat myotubes. A more in-depth analysis of the factors that are present in the extracts should provide further information [80].

Crystalline lens regeneration is an example in which the regenerative cells appear through transdifferentiation of pigmented epithelial cells of the iris [99]. It is possible to aggregate pigmented epithelial cells of salamander iris from the dorsal iris, to implant these cells in an eye whose crystalline lens has been previously removed and to obtain the formation of a new crystalline lens in the aggregate. Pigmented epithelial cells of the ventral iris do not normally produce a crystalline lens, but the activity of the *Six-3* gene induces lens formation through the transdifferentiation of ventral cells. Inhibition of the signaling pathway of the bone morphogenetic protein (BMP) also leads to induction of crystalline lens formation [100]. There is the theory that the BMP signaling pathway maintains ventral identity and that the cells assume a dorsal identity after its inhibition.

Planarian regeneration appears to occur almost exclusively through the contribution of neoblasts, although the current evidence does not necessarily exclude a contribution of differentiated cells [58]. In hydra regeneration, the activity of the epithelial stem cells implies budding and regeneration [101-102]. Studies with the jellyfish *Podocoryne* showed an example of transdifferentiation and its regulation by signaling [58]. When portions of striated muscle were explanted, they could be activated by enzymes that degrade the extracellular matrix, and that are later differentiated into smooth muscle and other types of cell. The impression left by these studies is that control of the differentiated state is prepared to allow considerable plasticity, which may be related to the important role of agamic reproduction and regeneration in these animals [58].

In view of evolutionary and biomedical problems, it is interesting to compare the relevant examples of plasticity in myotubes and in the pigmented epithelial cells of Urodeles and mammals. Although salamanders are the only adult vertebrates that can regenerate their crystalline lens, the pigmented epithelial cells of several vertebrates can be converted into crystalline lens cells in culture. It will be necessary to identify the blastemal signals that trigger these responses from differentiated cells, as well as the molecular basis of any differences between the differentiated cells of the two species. It is already evident that this approach to regeneration – i.e., the investigation of the plasticity of differentiated cells – is a productive and informative study, particularly in the absence of a complete genetic analysis [80].

Nowadays, the main method for regenerative medicine in mammals is the isolation of stem cells, followed by manipulations that aim to direct differentiation towards the morphogenesis of complex structures. Although this is attracting considerable interest at present, many of the applications are sufficiently problematic to justify the consideration of alternative and supplementary approaches. The strategy in Urodeles – the limited re-specification of residual differentiated cells – is so successful that it would come as a surprise if it were not potentially regarded as a therapeutic approach in some regeneration contexts in mammals. The example of myoseverin shows that the responses that are discussed in this review are a potential target for therapies geared towards regulating the stability of the differentiated state [80].

Finally, in theory, differentiated cells could also contribute to the formation of the blastema, without losing their differentiation status. This would apply to cells such as fibroblasts. Fibroblasts are mature cells that synthesize the extracellular matrix, but still maintain some of the characteristics of progenitor cells, such as elevated motility and proliferation capacity. Fibroblasts can be regarded as one of the main contributors to the blastema and they can achieve this without the occurrence of dedifferentiation [62]. Therefore, the processes that lie behind the formation of the blastema and cell origin are still being studied.

4. Cellular differentiation and environmental insults or stress

The cells are susceptible to changes arising from normal physiological processes as well as from changes in the external environment units. To avoid subsequent damage to these cells, there are homeostatic mechanisms that enable the adaptation of cells and tissues to changing conditions. Such mechanisms are activated not only under physiological conditions, but also to limit the damage imposed in response to injury and disease processes. Hence certain factors that may be present in the tissue microenvironment can lead to cellular stress, which in turn triggers mechanisms involving cell survival and adaptation strategies as well as injuries and consequent pathological process, or even cell death [103].

Stress-inducing conditions caused by changes in the cell microenvironment may affect the eukaryotic cells exposed either acutely, when they occur for a transitory period, or chronically, when they persist for a long time, prompting the cells to respond in a number of different ways, e.g., changing their metabolism, secretion and gene expression etc. [104].

Stress-inducing agents include thermal shock caused by hyperthermia or hypothermia [105, 106], osmotic shock [107, 108], nutrient [109–111] and ATP depletion, low oxygen concentration and oxidative stress [112, 113], changes in cell metabolism induced by cancerous transformation, mechanical stress, DNA damage due to exposure to UV radiation, baric stress and others. Therefore the cells may be exposed to a wide variety of agents, which will trigger different responses, affecting their function, activity and differentiation [103].

Once exposed to stress agents, the initial response of cells is generally a decrease in the synthesis processes of macromolecules such as normal proteins, RNA, DNA and fatty acids, and an increase in the synthesis of proteins belonging to a specific group called stress proteins,

probably regulated by changes occurring in the cytoskeleton during stress. These proteins are associated with the mechanism of cell cycle control, biosynthesis reactions and processing, including protein folding and oligomerization, translation, secretion, and repair of damaged proteins, and oxidative function [104]. This group of proteins is evolutionarily conserved, is present in the Archaea, Eubacteria and Eukarya domains, including the human proteome, and includes the so-called heat shock proteins, or Hsp, thus named because they were initially observed by Ritossa in 1962, due to the rise in temperature, but are also associated with other types of stress. Their high level of conservation suggests the performance of an essential role in cell metabolism [105].

The Hsps are part of the large group of proteins known as molecular chaperones, and have the ability to interact reversibly with other proteins, assisting in formation, folding and transmembrane transport [116]. In adverse conditions, such as increased temperature, osmotic or oxidative stress, Hsp levels are increased, remodeling the proteins that suffered injury, activating the synthesis and maturation of new proteins that will replace those affected by metabolic stress [117], or allocating them to an adequate proteolytic system, facilitating their elimination when the damage is not reparable [118]. The Hsps can be classified according to their molecular weight into five families or groups: HSP100, HSP90, HSP70, HSP60, and low-molecular-weight HSPs between 12 and 43 kDa [119].

Stress may also result in epigenetic modifications involving DNA and histone methylation, phosphorylation and acetylation, which change the expression of the genes without altering the DNA. Genomic changes may occur due to structural rearrangements and these alterations can be passed on to daughters and may lead to some changes which can be maintained in many cell generations [103].

The HSPs may interact with different apoptotic proteins, and can block essentially all apoptotic pathways, most of them involving the activation of cysteine proteases called caspases [120]. Some mediator proteins may alternate adaptive responses and direct the cells to apoptosis due to more severe stress, when this causes, for instance, DNA breakages. This mechanism involves checkpoint kinase 2 (Chk2) phosphorylation via p21 with cell cycle arrest and the action of proapoptotic p53 genes, activating apoptosis and cell death. Thus the cells may respond to stress, depending on its level and intensity, in various ways ranging from the activation of survival and adaptation pathways in milder situations, to channeling to cell death, in the case of more severe damage [103]. The degree of susceptibility to environmental changes varies in different cell types. Brain neurons, for example, are extremely sensitive to changes in their environment, dying quickly when environmental conditions are beyond the normal physiological patterns. On the other hand, fibroblasts exhibit extreme resistance to damage, surviving drastic metabolic changes, such as the complete deprivation of oxygen.

The characteristics and functions of stem cells make them particularly susceptible to certain stress factors such as oxidative stress, mechanical stress, growth factors, and cytokines signal affecting the cell differentiation and division regulation mechanisms. The long lifespan and high proliferative potential of stem cells makes them prone to cellular transformation (cancer), and the stress responses that lead to senescence and apoptosis can in fact act as anticancer

protection mechanisms, by preventing the continuity of the cell division process, thus limiting their lifespan [121].

4.1. Stress proteins, differentiation and cancer

During cellular transformation in tumor growth, the cells are experiencing drastic shifts in their intracellular and extracellular environment, because they are frequently exposed to stress conditions that include hypoxia, acidosis, nutrient deprivation and immune system attacks. Accordingly, the tumor cells must be able to adapt to a variety of stress conditions to survive and proliferate, so these stress condition may act as a driving force behind evolution (oncogenesis) and adaptation (acquired treatment resistance) [122]. In various types of tumor, the expression of molecular chaperones including the Hsps is increased, reflecting an effort of the tumor cells to maintain tissue homeostasis in an unsuitable environment. This mechanism can facilitate the survival of tumor cells from these injury conditions, thus representing a possible selecting agent for further mutations, and the management of the oncogenic process [123].

However, the Hsp levels are useful biomarkers for carcinogenesis in some tissues and signal the degree of differentiation and the aggressiveness of some cancers. Therefore, these levels can be useful as an adjunct in tumor diagnosis, but are not informative at the diagnostic when considered alone. Several Hsps are implicated with the prognosis of specific cancers; for example Hsp70 is correlated with poor prognosis in breast, endometrial, uterine, cervical, and bladder carcinomas, while Hsp27 expression is associated with poor prognosis in gastric, liver, and prostate carcinoma, and osteosarcomas. On the other hand, increased Hsp expression may also predict the response to some anticancer treatments. For example, Hsp27 and Hsp70 are implicated in resistance to chemotherapy in breast cancer, Hsp27 predicts a poor response to chemotherapy in leukemia patients, while Hsp70 expression predicts a better response to chemotherapy in osteosarcomas [123]. As Hsp70 and Hsp27 are the most active chaperones in the subversion of the programmed cell death pathway (apoptosis) and play major roles in cancer, because these chaperones are also able to inhibit senescence pathways (e.g., p21 and p53 dependent), allowing cell division limits to be surpassed [119,124], these implications of Hsp in tumor progression and response to therapy have led to the possibility of Hsp use in anticancer vaccines, exploiting their ability to act as immunological adjuvants [125,126].

Based on the fact that most cancer cells abundantly express HSP70 at the basal level to resist various insults at different stages of tumorigenesis and during anti-cancer treatment, some studies have investigated HSP70 and HSP90 inhibitors for their use in anti-cancer therapy [127,128].

5. Conclusion

The goal of regenerative medicine is to restore the cells, tissues and structures that are lost or damaged after disease, injury or aging. Regenerative medicine currently uses three main approaches (4): the use of stem cells to build new structures, the implantation of cells preconditioned to differentiate in a particular direction, and the stimulation of endogenous cells to

replace structures that have disappeared. In these strategies, we can choose to use or disregard biomaterials with carrier and/or stimulator agents. The regeneration of organs and limbs that occurs in several groups of animals provides another important viewpoint, as it demonstrates that under some conditions, complex adult tissues can be rebuilt. Biological regeneration lessons have not been widely assimilated, partly because this attribute seems minor when we focus on mammals.

It is not understood why some animals are able to regenerate and others are apparently not, but even from our current limited perspective, there appears to be a series of differences between mammals and Urodeles that prevent or limit regeneration. One of the aspects of wound healing in adult mammals is the reduction of the regenerative capacity and the occurrence of fibrosis and inflammatory responses [83].

We understand that it is necessary to glean a better idea of the cell differentiation, transdifferentiation, dedifferentiation and redifferentiation mechanisms that occur in different contexts, such as during regeneration in animals. The participation of stem cells and progenitor cells in this process is also of considerable relevance. The understanding of these mechanisms can facilitate highly relevant therapeutic strategies in regenerative medicine and tissue engineering.

Gene/Factor	Effect/Importance
pRb	Essential in regulating G1-phase S transition. Evidence indicates major importance for the maintenance of the state of differentiation in vertebrate myotubes.
Prothrombin	Activated selectively on the dorsal margin of the iris after injury. Generator of thrombin protease.
Thrombin	Activates the coagulation cascade and other events to mediate the response to the injury.
<i>Prod 1</i>	Encodes a small protein that is bound to the cell surface by a GPI anchor. Apparently analogous to the mammalian CD59 protein, implicated in positional identity determination.
<i>Nag</i>	Identified as a binder to <i>Prod 1</i> . Acts as a mitogenic factor for blastema cells.
β-catenin	Signaling pathway for the regenerative process in the formation of the <i>Hydra</i> head. Participation in regeneration in planaria.
<i>Pax-7</i>	Expressed from the activation of muscle satellite cells, during the regeneration process in tritons.
p16^{INK4}	Causes an impediment to cell cycle reentry.
Homeobox Gene <i>Msx-1</i>	Promotes cell proliferation. Its expression is inversely correlated to differentiation. It has been proposed that <i>Msx-1</i> is a master regulator of the program for cellularization in regeneration in Urodeles.
<i>Myoseverin</i>	Depolymerizes microtubules, an activity observed in myotubes of mammals. Induces changes in the expression of genes involved in repair, wound healing and regeneration. There is a theory that <i>myoseverin</i> can activate the expression of a program that acts on cellularization and other functions that are relevant to regeneration;
<i>Six-3 Gene</i>	Induces the formation of the crystalline lens through the transdifferentiation of ventral cells of the iris in mice.

Gene/Factor	Effect/Importance
Bone Morphogenetic Protein (BMP)	The inhibition of its signaling pathway leads to the induction of crystalline lens formation. It can maintain ventral identity and the cells assume a dorsal identity after its inhibition.
RA gradient	Operates in the development of rat limbs, as a consequence of its synthesis, close to the midline of the animal and its degradation by the product of the CYP 26 gene, expressed at the distal extremity of the limb bud.
Canonical Wnt signaling pathways	Involved in the appendage regeneration process
Fibroblast growth factor (FGF)	Involved in the appendage regeneration process.
Signaling of activin; retinoic acid; non-canonical Wnt.	Involved in the regulation of regeneration in the sea urchin.
SALL4	Involved in the maintenance of pluripotency. Expressed during blastema formation.
ATF3, JUN3, EGR1, NR4A2 and FOS	An increase is observed in these oncogenes during the epithelialization and blastema phases.
MSP1	Mitotic kinase essential for cardiac regeneration in the zebrafish.
GATA4	Heart development transcription factor essential for cardiac regeneration in the zebrafish.

Table 1. Some biochemical parameters to the regenerative responses and their consequences.

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