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REVIEW

Regeneration puzzle: correlations of immunity and DNA methylation in regenerative annelid models

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Abstract

Distinct gene expression patterns are important to various biological functions, spanning developmental processes, wound healing, and the restoration of body parts. Moreover, immunity intertwines with these processes, as researchers propose links between immune system evolution and the variable regenerative capacities seen across different organisms. Concomitantly, elements that influence gene expression can also affect regeneration, since DNA methylation is a key epigenetic mechanism that emerges as a critical regulator of cellular fate and behavior. While various studies propose methodologies for detecting and quantifying DNA methylation under diverse experimental settings, its interaction with regeneration remains relatively unexplored, particularly in annelids. This review aims to address this gap through exploring the connections between immunity, regeneration, and epigenetics by compiling information from studies conducted in different organisms and focusing on annelids as regenerative models. Additionally, it also provides an overview of protocols applying monoclonal antibodies to target specific DNA methylation forms.

Key Words: invertebrates; inflammation; restoration; epigenetics; immune response

Introduction to the regenerative machinery

Many invertebrates and some vertebrates share the astounding capacity to restore body parts that are amputated or injured. Interestingly, the evolutionary loss of regenerative ability in higher organisms is commonly associated with the development of the adaptive immune system. These observations increase the conjectures concerning the possible roles of immunity in regeneration (Mescher *et al*., 2017; Arinda *et al*., 2022).

Tissue restoration, regeneration, and inflammation are part of the normal homeostasis in adult metazoans. Curiously, certain animal species readily regenerate their lost body parts, while others do not; however, the background of this dichotomy is still obscured (Brockes and Kumar, 2008). As a response to wounding, several cellular and molecular mechanisms are initiated to reconstruct damaged or lost body parts or organs. Three major processes are involved in tissue restoration as post-embryonic morphogenesis: wound healing, tissue repair, and regeneration. This review emphasizes the comparative aspects of inflammation and epigenetics in the course of regeneration, rather than solely listing the similarities and differences of regenerative machinery in invertebrates and vertebrates.

Regeneration is an incredibly complex process, defined as an adaptive trait, which explains its great diversity among closely related species. The capacity to regrow functional body parts is immensely affected by the type and place of injury, as well as by the environment surrounding the organisms subject to regeneration (Chen and Poss, 2017). In adult individuals, regeneration is part of postembryonic morphogenesis and can be seen as a byproduct of development, at the same time that it implies mechanisms not involved in normal developmental processes (Brockes and Kumar, 2008). Both invertebrate and vertebrate organisms express variable regenerative potential, which is influenced by transcription factors, signaling pathways, nerve supply regeneration, bioelectrical signals, production of reactive oxygen species (ROS), epigenetic machinery, as well as the immune system (Brockes *et al*., 2001; Brockes and Kumar, 2008; Chen and Poss, 2017).

Before a regeneration program is initiated in the cells, wound healing is necessary, which includes modifications in cell metabolism and migration. Concomitantly with the wound closure and healing

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process, the loss of cell-cell adhesions and modification of the extracellular matrix occur, leading to indirect activation of proliferation and dedifferentiation pathways in the tissues surrounding the wound (Xiao *et al*., 2011; Kostyuchenko and Kozin, 2021). Subsequently, tissue restoration can arise through two main categories of processes: morphallaxis and epimorphosis. In morphallactic regeneration, pre-existent tissues are remodeled to form the missing structures. On the other hand, during epimorphic regeneration, cells proliferate to construct a blastema, which later will grow and differentiate into functional segments to replace the missing ones (Özpolat and Bely, 2016). In most cases, however, a successful tissue restoration is a combination of morphallactic and epimorphic processes (Kostyuchenko and Kozin, 2020).

Certain invertebrates (cnidarians and planarians) have vast regeneration capacity to restore their whole body following traumatic injury. Cnidarians (based on the experimental information derived from *Hydra*) can re-organize their body even from single, dissociated cells (Reddy *et al*., 2019). Recent experimental information claims that regeneration in *Hydra* is maintained by stem cells activated through injury signals and those are engaged in the pattern formation of body restoration (Holstein, 2023). During regeneration of the planarian *Schmidtea mediterranea*, its neoblasts (planarian stem cells) actively divide to re-organize the injured organism (Peiris *et al*., 2014). Interestingly, among *Ecdysozoa* the regeneration capacity is limited to cellular (axon regeneration in *Caenorhabditis elegans*) or appendage levels (limb restoration in crickets) (Nix *et al*., 2014; Bando *et al*., 2022). In various annelid groups, different regeneration capacities can be observed: oligochaetes can restore a certain number of segments (with their internal organs) along their body axis, while leeches have a regeneration capacity restricted to muscle cells and neurons (Schikorski *et al*., 2008; Bely 2010). Regardless of the partly explored regeneration capacity of deuterostome invertebrates, such as tunicates, echinoderms, hemichordates, and cephalochordates, their restoration abilities vary from cell to whole organism extent (Ferrario *et al*., 2020).

Among vertebrates, urodele amphibians, such as salamanders and newts, have to be mentioned due to their marvelous capacity to restore many body parts: not only limbs or tail, but lens, brain, and heart. Additionally, organ regeneration can be exceptionally exemplified by the zebrafish (*Danio rerio*), which is able to restore complete organs, such as the heart, pancreas, kidney, and liver, besides structures of the nervous system. Taking a step further, most amniotic animals have lost regenerative capacity, besides some exceptional examples, like ear regeneration in spiny mice or digit tip restoration in newborn humans (Brokes and Kumar, 2002; Mescher *et al*., 2017; Daponte *et al*., 2021).

Comparative aspects of inflammatory response in tissue restoration

Various exogenous and endogenous factors can induce regeneration programs upon injury. First of all, recognition of injury must be started, followed by wound healing and activation of a single or a combination of tissue restoration programs. Wound healing is a general process described in every animal, but only in some species it can induce a regenerative response, while in others it triggers scar tissue formation (Abnave and Ghigo, 2019). What remains to be answered is what cellular or molecular events can trigger regeneration instead of scar formation. Studies (Schikorski *et al*., 2008; Bando *et al*., 2022) have suggested the involvement of cell signaling pathways – such as the activation of Toll/Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs), as well as signal transduction through JAK/STAT cascade – in the modulation of the injury environment for recruitment of immune cells and later activation of cellular programs that lead to regeneration.

Mechanisms of innate immunity are conserved from invertebrates to vertebrates: it is thought that some vertebrates can only have regenerative capacity because of a balance between their innate and adaptive immune systems. Because the immune response upon an injury frequently leads to the formation of fibrosis and scarring, which significantly hinders the regeneration process, a counteract is necessary to allow the restoration of anatomical structures and keep their functionality, and this step is performed by macrophages or macrophage-like cells (for example, hyaline amoebocytes in the case of annelids) (Galliot *et al.*, 2017; Bodó *et al*., 2021; Bando *et al*., 2022).

The innate immune system of invertebrates shares similarities with vertebrates from the aspect of cellular components in comparison to macrophages in both morphology and function. However, taking earthworms as an example, even presenting only an innate immune system, they exhibit significant capacity to regrow body segments with differentiated phenotype and function equivalent to the original tissues. Nevertheless, the outcome of the restored structures depends on the amount and location of tissue lost, within other factors (Bodó *et al.*, 2021). On the other hand, higher organisms with well-evolved adaptive immunity have restricted or absent regenerative capacity, like most mammals (Bely, 2010; Rennolds and Bely, 2023). An evolutionary perspective suggests that this phenomenon occurs on account of the necessity for quick defenses instead of a long-term activity. In this scenario, regenerative capacity appears to have an inverse correlation with the evolution of the immune system (Julier *et al*., 2017; Bodó *et al.*, 2021).

For instance, closely related species of invertebrates have great variability in the restoration of body parts (Bely, 2010). This capacity also changes depending on the body sites of a single individual (eg. comparing anterior and posterior

segment regeneration in annelids). This occurrence might be related to the complexity of systems and structures located in different body regions, as well as the hypothesis of the immune and nervous systems of annelids taking part in the modulation and induction of total segment restoration (Molnár *et al*., 2015). Besides, the developmental stage or age of the organism can also affect how the immune system reacts and induces healing and regeneration (Xiao *et al*., 2011; Cooke, 2019). After an injury, the body has to recognize the loss of tissue, so that wound healing and regenerative programs can be started (Rennolds and Bely, 2023). The activation of a regenerative set-up depends on a range of signals, which vary depending on the organism, whilst the continuation of this effect - leading to the restoration of structures - is connected to the modulation of the immune system. With that in mind, it is possible to think that the issue is not only finding a correlation between injury and induction of regeneration but also suggesting what can inhibit or hinder regeneration upon injury (Bhambri *et al*., 2018).

Generally, the immune system influences the outcome of the healing process after damage or loss of tissue, determining if there will be a regenerative process or not. Fundamentally, the role of the innate immune response against an injury is to cause an inflammatory signaling cascade that modulates cell plasticity, leading to different levels of regenerative capacity (Brokes and Kumar, 2002). In a general approach, tissue damage induces the release of signaling molecules that trigger cell signaling pathways. Their major outcome is the production of cytokines, chemokines, and antimicrobial mediators, which produce an innate inflammatory response and impact cell proliferation, survival, and apoptosis, being an important influence on tissue regeneration (Schikorski *et al*., 2008). Moreover, any element that interferes with gene transcription, such as DNA methylation, might be a concern regarding the expression of the inflammatory processes and molecules caused by the signaling pathways activated after injury (Julier *et al*., 2017).

One of the hypotheses is that this type of response can be triggered by damage-associated molecular patterns (DAMPs), recognized by various sets of pattern recognition receptors (PRRs); their analogs have been identified in several organisms along different animal groups (Škanta *et al.*, 2013). This causes the transduction of signals that culminate in NF-κB activation and expression of inflammatory cytokines and antimicrobial factors. Such an inflammatory state contributes to the injured tissue and its surrounding cells with the phenotypic fluidity that allows regeneration (Cooke, 2019).

Activation of the immune system with the recruitment of specific cell types and the consequent production of cytokines and other molecules are needed and crucial for regeneration in various organisms. However, exacerbated or persistent activity of the immune system - in the form of inflammation - can modulate the regenerative potential in certain issues or cell populations, giving rise to undesired scenarios (Stein and Cooper, 1983; Abnave and Ghigo, 2019). Hypotheses suggest that the amputation of body parts can cause the reprogramming of differentiated cells into proliferating stem cells (Duncan and Alvarado, 2019). Therefore, it is possible to assume that mechanisms influencing cell fate and reprogramming, such as epigenetic marks, can affect the gene expression dynamics during development and regeneration in different organisms. It is suggested that it happens in order to induce or inhibit the establishment of a pluripotency or proliferation state (Kostyuchenko and Kozin, 2021).

Earthworm regeneration and immune response

Annelid earthworms are particularly interesting from the point of view of regeneration. *Eisenia andrei*, like the other members of the Oligochaeta subclass, has anatomical features that aid its regenerative capacity: a body that is made of segments (annulations). Each segment is linked via the coelomic cavity, which is in contact with every organ in the earthworm's body, through coelomic fluid and cells, called coelomocytes (Hostetter and Cooper, 1974). Besides that, the expression of *Hox* genes homologs for anterior-posterior axis definition is involved in the later stages of segment diversification, performing a role that connects normal development and regenerative programs (Shankland and Seaver, 2000).

One of the exceptional characteristics of the coelomic cavity is the diversity of coelomocytes, originating from the mesenchymal layer of the cavity, that can migrate and contact to any structure of an earthworm. These cells can be considered as the effector cells of such organisms, and their subsets take part in different processes. Several types of coelomocytes are morphologically described, but two major groups play an important role in immune responses upon injury: eleocytes and amoebocytes (hyaline and granular subtypes) (Cooper, 1996; Bilej *et al*., 2010; Engelmann *et al*., 2016).

The coelomic cavity of earthworms is considered the antecedent form of the body cavity (peritoneum and pleura) of vertebrates, and this can be justified by the fact that the cells and molecular factors that compose it are in contact with all organs and systems of the earthworm's body. Therefore, the presence of a coelomic cavity with such features allows the execution of cellular and humoral immune functions, such as wound healing, graft rejection, elimination of pathogens (by phagocytosis and encapsulation or/and by the aid of antimicrobial factors), and in some cases, it plays a role in the development of a spectrum of regenerative capacity (Hostetter and Cooper, 1974; Homa *et al*., 2013; Engelmann *et al*., 2016).

To recognize pathogens or damaged cells the innate immune system operates with a handful of molecular factors, the PRRs. By now, several PPRs have been identified in earthworms (Prochazkova *et al.*, 2020), however, their exact molecular interactions with ligands and their protein expression profiles are rather unclear. Among those, the single cysteine cluster (scc) and multiple

cysteine cluster (mcc) TLRs have been characterized in *E. andrei* earthworms and that is concordant with most of the previously described protostomian and deuterostomian TLRs (Prochazkova *et al*., 2020). According to recent data (Bodó *et al.*, 2021; Aigner *et al*., 2023), TLR signaling is also involved to some extent in wound healing and regeneration of earthworms.

All coelomocytes participate in the wound closure by forming a wound plug. Both amoebocytes and eleocytes take part in events throughout wound healing and regeneration. Hyaline amoebocytes are macrophage-like cells that play a role in epidermis regeneration during wound healing and simultaneously create an inflammatory reaction (Cooper, 1996). The segments that form an earthworm's body are interconnected not only with each other through the coelomic cavity, but also with the exterior environment through dorsal pores. Such structures allow the secretion of coelomic fluid and cells, as well as the entry of pathogens and contaminants. Therefore, this way of interaction is considered crucial for the activation and development of immune responses in earthworms (Bilej *et al*., 2010), including a probable contribution to the regeneration of body segments.

Taking wound healing and inflammation in annelids as an example, undifferentiated mesodermal cells migrate to the wound site to seal it, forming a mass capable of differentiation and proliferation, denominated as blastema (Fig. 1). This will be the tissue of origin for the segments to be restored, and its development experiences interference from cells of the coelomic cavity, especially macrophage-like hyaline amoebocytes (Abnave and Ghigo, 2019). Blastema formation occurs through the accumulation of a mass of undifferentiated cells under the epithelium; these cells are transcriptionally highly active for *Hox*, *Wnt*, and GMP genes (germline/multipotency program), promoting proliferation (Stein and Cooper, 1983; Özpolat and Bely, 2016;).

According to a study performed by Zheng *et al.* (2016), stem cell pluripotency factors (*Sox2, Oct4, Klf4, nanog, c-myc*) are upregulated during regeneration of posterior amputated segments in *Eisenia fetida.* Such factors are crucial for embryonic pluripotency, participating in self-renewal and transcriptional regulation; however, different expression patterns are obtained depending on the body site and time after amputation. This information reinforces the hypothesis that regeneration in annelids – and maybe in other invertebrates – happens by cell dedifferentiation and reprogramming (Zheng *et al*., 2016; Shao *et al*., 2020).

In another example, Planques *et al.* (2021) investigated the effects of DNA methylation in a different invertebrate model, in the polychaete annelid *Platynereis dumerilii*, suggesting that gene body methylation (especially in the form of 5-methylcytosine) and the expression of stem cell markers (such as Nucleosome Remodeling and Deacetylase, *NuRD* complex) are mainly present in undifferentiated proliferating cells of the growth zone of amputation sites. This suggests the need for a stem cell-like expression system to aid tissue restoration (Planques *et al*., 2021). In addition, Aigner and colleagues (2022b) refer to gene body methylation (gbm) as more commonly described in invertebrates, where it directly affects transcriptional activity. Data indicate the occurrence of gbm in *Lumbricus terrestris* as another annelid model, but the same approach to evaluate DNA methylation is not yet described in *Eisenia* species.

DNA methylation during regeneration

The role of epigenetic mechanisms is substantially described in vertebrates, influencing many aspects of development, cell behavior, and proliferation in homeostasis and pathological conditions. With regard to DNA methylation, its modified patterns are reported as relevant during transitions of developmental stages of vertebrates. Previous studies have evaluated the effect of hypomethylating agents on the development and regeneration of different organisms as well (Planques *et al.*, 2021). Therefore, we consider the hypothesis of a variable DNA methylation pattern occurring in the course of the regeneration process, in this case, using invertebrates (annelids) as animal models, once they exhibit great capacity to restore complex body parts.

Epigenetic marks participate in development and tissue differentiation by securing a specific gene expression program to define cell fates, being affected by a diverse range of factors intrinsic to the organism and respective to the environment (Allis and Jenuwein, 2016). Control of gene expression is crucial to regulate cell proliferation and differentiation, which has a pivotal role in regeneration. Because one of the main steps for regulation of gene expression happens during DNA transcription, the modification of any component of the transcriptional machinery can affect if and how genes will be transcribed in a conventional fashion or in an altered way. Elements that can undergo changes and interfere with cell behavior might ultimately affect development and regeneration, such as transcription factors, chromatin remodeling, and DNA methylation (Kanherkar *et al*., 2014).

Even though the enzymes participating in DNA methylation and demethylation processes are well conserved between vertebrates and invertebrates, there are significant variations in methylation patterns when comparing these groups of organisms. They can present very different levels of global DNA methylation, as well as variable sites for this type of nucleotide modification (Hendrich and Tweedie, 2003). DNA methylation can be associated with gene activation or silencing, which occurs mainly through the modulation (induction or inhibition) of transcription factor binding. Different sites and amounts of methylation can lead to different outcomes, whether it is in a gene promoter or gene body region. In addition, the variable positions of DNA methylation in different nucleotides can interfere with the behavior of the transcriptional machinery (Planques *et al.*, 2021).

In both invertebrates and vertebrates, distinct nucleotides can be targets of methylation in either DNA or RNA, and the positions of the covalent addition of methyl groups can also vary within a

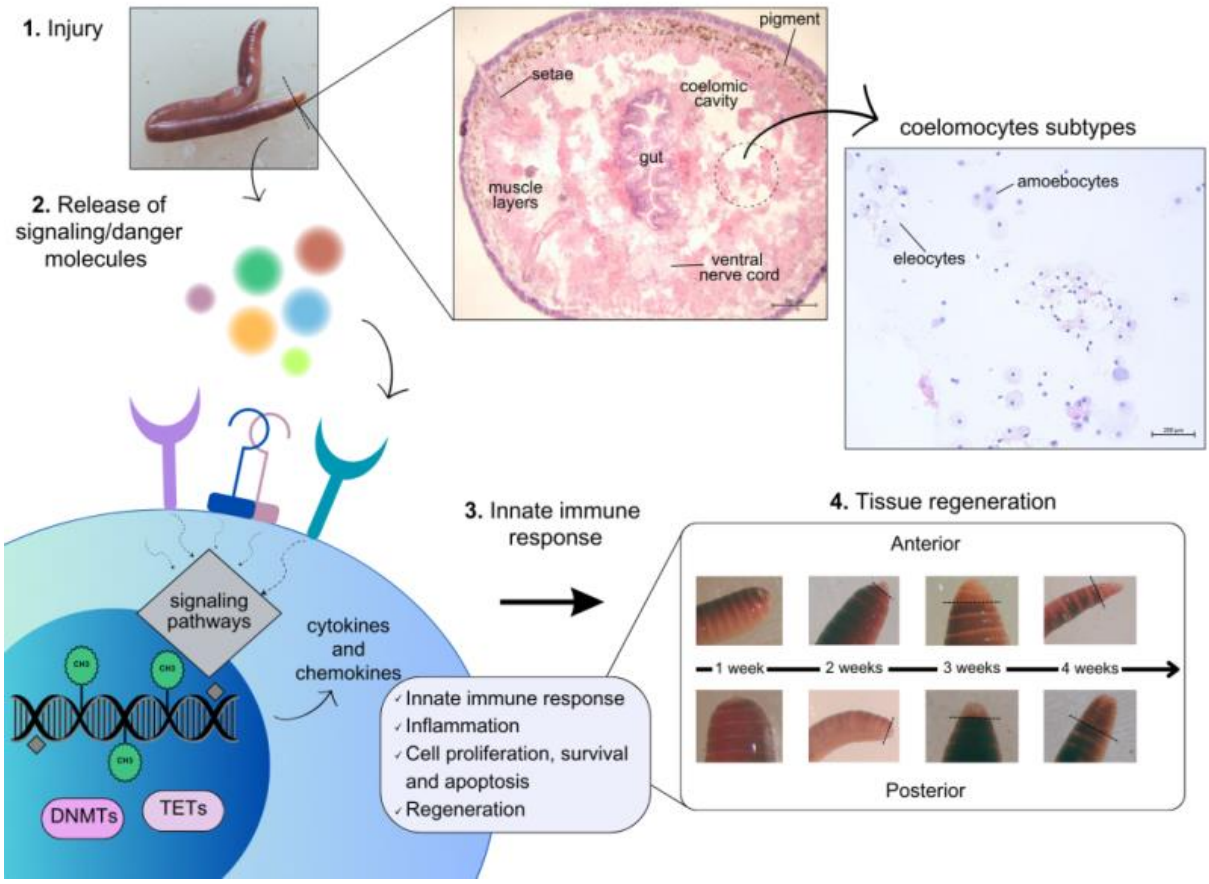

Fig. 1 Overview of earthworm segment regeneration and possibly involved subsequent events: injury, signaling molecules/intracellular signal transduction, epigenetic mechanisms, and innate immune response. The left bottom side presents cellular phenomena that can affect injury-induced inflammation. The right upper side shows subtypes of coelomocytes that play a role in immune response and might interfere with the regeneration process. The right bottom corner exhibits a timeline of anterior and posterior regeneration of the *Eisenia andrei* earthworm, showing the regenerating blastema (separated from the original segments by dashed lines)

single nucleotide. For instance, cytosine is the most methylated nucleotide, but its effect on gene transcription can vary if the product of methylation is 5-methylcytosine (5mC) or 5-hydroxymethylcytosine (5hmC), depending on the enzymes involved in methylation and demethylation reactions, as well as the position of the methyl group attached (Breiling and Lyko, 2015). Additionally, adenine is another nucleotide that can have the binding of methyl groups in both DNA and RNA molecules, but its occurrence is generally less described. Furthermore, the same form of methylation can cause variable outcomes in different organisms by either gene activation or silencing (Fernandes *et al.*, 2021).

Given the clear role of epigenetics – particularly of DNA methylation – interfering with gene expression and impacting cell behavior, reprogramming, and differentiation (both in developmental and regenerative states), certain questions can be raised: can we observe changes in the methylation pattern during different periods of regeneration? Does the modulation of DNA methylation modify the regenerative capacity of an organism? How is the activity of the methylation and demethylation enzymes throughout regeneration?

In recent years, an increased number of studies (Regev *et al*., 1998; Lunyak and Rosenfeld, 2008; Katsuyama and Paro, 2011; Rasmussen and Helin, 2016; Planques *et al*., 2021; Aigner *et al*., 2022a; Boulias and Greer, 2022;) have been directed to identify the roles of epigenetics in physiological and pathological conditions in a range of different organisms. To analyze those factors in the scenario of regeneration, it is necessary to have a model not only able to regenerate body parts efficiently, but that also has the targeted methylation forms spread throughout its body in levels that can be detected. These circumstances are directly connected to the methods we possess nowadays to detect and quantify DNA methylation tags, as well as the crucial need to optimize these methods for the samples we apply in these studies.

From the perspective of global methylation assessment, without a gene-specific approach, it is possible to use immunological methods based on

monoclonal antibodies to localize and quantify different sites of DNA methylation. Immunohistochemical studies can be used for qualitative evaluations, still allowing semi-quantitative interpretation. Monoclonal antibodies raised against certain methylated nucleotides can be applied to tissue sections: in the immunohistochemistry technique, they signal structures, organs, and systems that present variation in specific forms of DNA methylation and can be used to compare intact and regenerated tissues, for example. However, in most cases, the binding of these antibodies to their target antigens requires pre-treatment of the samples, once the epitope is located within the DNA structure. Nevertheless, the same monoclonal antibodies can be applied to dot blot assay for a quantitative investigation, yet in a global-methylation manner.

A complementary approach is the evaluation of the enzymes involved in methylation and demethylation processes and their enzymatic activity. In this scenario, the targets of analysis are the isoforms of DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) hydroxylase enzymes. Commercially available nuclear extraction kits and colorimetric kits for their analysis facilitate this approach (Planques *et al*., 2021; Aigner *et al*., 2022b). Although the occurrence of DNA methylation is maintained throughout many species, there is not enough information to confirm how conserved the related enzymes (DNMTs and TETs, for example) are, especially in earthworms. Nonetheless, hypomethylating agents such as Azacytidine and Decitabine, which interfere with the function of the aforementioned enzymes, are used in different experimental animals and cell lines. Their administration modulates levels of DNA methylation, and experiments have evaluated their effect on some processes, including development, regeneration, and post-regeneration growth. In the studies of Manzoni *et al.* (2016) and Sajadian *et al*., (2015), mammalian cell lines exposed to those drugs presented a reduced amount of global DNA methylation, together with an increased expression of TET2 enzyme, suggesting that both passive and active demethylation processes take place.

Furthermore, the employment of hypomethylating drugs was also performed in annelids. In a study by Planques *et al.* (2021), Decitabine caused the reduction of 5mC levels in *P. dumerilii*, as well as impairment of regeneration and post-regeneration growth could be observed in the experimental model. Moreover, Aigner and collaborators (2022a) observed that Aza (5-aza-2'deoxycytidine) did not alter global DNA methylation levels in *L. terrestris*, and its effect on DNMTs and TETs isoforms expression was not significant within different samples.

Detection of DNA methylation through immunological methods: protocol optimization by DNA denaturation and antigen retrieval

The employment of immunological methods to quantify or localize DNA-bound antigens (methylation tags, in this case), requires the antigens in question to be available to bind antibodies. Therefore, the interactions of tissues and DNA structure with the reagents used for sample preparation have to be considered. During the fixation of histological samples (paraffin-embedded or cryosections), the application of formaldehyde-based fixatives effectively preserves the tridimensional nuclear conformation of nucleic acids. Although this is extremely important for assessing the antigens localized within the DNA molecule, formaldehyde-based fixation can increase the complex form of proteins with calcium ions, causing antigen masking (Morgan *et al*., 1994).

The ‘calcium theory’ proposes that calcium ions have a role as promoters of antigen masking, which is proven valid for protein antigens. Besides this theory, a different viewpoint is that methylation tags recruit by themselves masking proteins, which would inhibit the binding of antibodies. The native chromatin conformation, the presence of MBPs (methyl-CpG-binding proteins), and formalin-induced crosslinks (methylene bridges) are suggested as other reasons that hinder the detection of methylation sites when using monoclonal antibodies (Çelik, 2015). Generally, tissue samples to which formalin-based fixation is applied and/or paraffin embedding process occurs can have epitopes modified by different reasons: heat, dehydration, or protein cross-linking, which cause antigen destruction, alteration, or masking. Heat-induced epitope retrieval (HIER) is commonly the preferred method to expose antigens in formalin-fixed sections, although its precise mechanism of action in the tissue is unknown (Krenacs *et al*., 2010).

Nonetheless, in the case of DNA-bound antigens, denaturation is the suggested intervention to expose the target epitopes. However, the technique should be able to preserve the tissue organization of the sections, as well as not cause the loss of DNA or its methylation tags, for example. For different antigens, heating the sample in the presence of salt enhances the detectability of DNA-bound antigens, while acidic denaturation protocols cause high background signal in immunohistochemical studies (Krenacs *et al*., 2010; Beaujean, 2018).

For that reason, Beaujean (2018) proposed the use of a citrate boiling solution to denature the DNA and expose epitopes in cryosections for further immunohistochemical application. Because citrate strongly interacts with calcium, these ions are released from the complex formed with proteins, unmasking the antigens. Similarly, Krenacs and collaborators (2010) also describe the citrate buffer as one of the most commonly employed for safe and successful HIER, applying a molarity between 0.01M and 0.1M at pH 6.0. Commercially available monoclonal antibodies raised against DNA methylation sites have selective affinity for methylated nucleotides on denatured DNA strands. Yet, there is no consensus about the ideal antigen retrieval protocols; neither is it recommended by the monoclonal antibody manufacturers.

The application of monoclonal antibodies for the assessment of DNA-related features also includes chromatin immunoprecipitation (ChIP) as a well-developed method (Das *et al.*, 2004). It allows to

identify or quantify certain proteins in a specific region of the genome; it can be applied in association with other methods, for example, amplification and identification by PCR (ChIP-PCR), sequencing (ChIP-Seq, ChIP-on-ChIP), and bisulfite methylation sequencing (ChIP-BMS). Those methods enable the evaluation and quantification of different DNA methylation forms when genomic (complete or targeted) sequences are available; otherwise, the assessment should target the detection of methylation enzymes with antibodies directly by ChIP (Li and Tollefsbol, 2011; Gavin *et al.*, 2012; Tabish *et al.*, 2019).

Concluding remarks

To the present day, considerable amounts of studies have hypothesized the inverse correlation between the development of an immune system and the capacity to regenerate body parts (Bely, 2010; Abnave and Ghigo, 2019). Plenty of studies (Katsuyama and Paro, 2011; Xiao *et al*., 2011; Planques *et al*., 2021; Paul *et al.*, 2022; Rennolds and Bely, 2023;) also contemplate other factors that might interfere with the regenerative ability shared – at different levels - between multiple organisms, in a scenario where epigenetic mechanisms are worth mentioning. In that matter, using invertebrates (such as annelids) as animal models allows an approach that combines less-developed immunity with higher regenerative ability. Adding epigenetic mechanisms – especially DNA methylation - as another variable to this analysis brings more questions about cellular and molecular events taking part in regeneration.

There is evidence pointing to an inverse correlation between the development of the immune system and the capacity to restore body parts, shown through several studies (Bilej *et al*., 2010; Bodó *et al*., 2021; Rennolds and Bely, 2023) employing various animal models. The investigation of invertebrates' immunity and correlating its findings with the characterization of their regenerative capacity has been enlightening in understanding the multiple mechanisms, events, and features underlying the highly variable capacity to restore body parts shared by many organisms. Combining such considerations with the everyday expanding area of epigenetic studies, also in multiple organisms, opens doors for questioning the relationship between these fields.

Nevertheless, questions remain. What is the specific function – if there is such – of epigenetic mechanisms, specifically of DNA methylation, in the regeneration process of invertebrates? If this correlation is valid, can the manipulation of epigenetic tags interfere with the capacity that certain organisms retain to regenerate? Can those hypotheses be extrapolated to higher organisms? Further experimentation is necessary to build up the bridges around this knowledge and clarify the possible answers to these questions. Concerning the experimental approach for this kind of research, optimizing methods for different sample types is of crucial importance to guarantee the collection of trustworthy data regarding the detection of epigenetic marks.

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References

Abnave P, Ghigo E. Role of the immune system in regeneration and its dynamic interplay with adult stem cells. Sem. Cell Dev. Biol. 87: 160-168, 2019.

Aigner GP, Nenning P, Fiechtner B, Šrut M, Höckner M. DNA methylation and detoxification in the earthworm *Lumbricus terrestris* exposed to cadmium and the DNA demethylation agent 5-aza-20-deoxycytidine. Toxics. 10: 100, 2022.

Aigner GP, Pittl V, Fiechtner B, Egger B, Šrut M, Höckner M. Common mechanisms cannot explain time- and dose-dependent DNA methylation changes in earthworms exposed to cadmium. Sci. Total Environ. 812: 151468, 2022.

Aigner GP, Peer V, Fiechtner V, Piechnik CA, Höckner M. Wound healing and cadmium detoxification in the earthworm *Lumbricus terrestris* – a potential case for coelomocytes? Front Immunol. 14: 1272191, 2023.

Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. Nat. Rev. Genet. 17: 487-500, 2016.

Arinda BN, Innabi YA, Grasis JA, Oviedo NJ. Non-traditional roles of immune cells in regeneration: an evolutionary perspective. Development. 149: dev199903, 2022.

Bando T, Okumura M, Bando Y, Hagiwara M, Hamada Y, Ishimaru Y, *et al*. Toll signaling promotes blastema cell proliferation during cricket leg regeneration via insect macrophages. Development. 149: dev199916, 2022.

Beaujean N, Salvaing J, Abd Hadi NA, Pennings S. Antibody-based detection of global nuclear DNA methylation in cells, tissue sections and mammalian embryo. Methods Mol. Biol. 1708: 59-80, 2018.

Bely AE. Evolutionary loss of regeneration: pattern and process. Integr Comp Biol. 50: 515-527, 2010.

Bilej M, Procházková P, Šilerová M, Josková R. Earthworm Immunity. Adv. Exp. Med. Biol. 708: 66-79, 2010.

Bhambri A, Dhaunta N, Patel SS, Hardikar M, Bhatt A, Srikakulam N, *et al*. Large scale changes in the transcriptome of *Eisenia fetida* during regeneration. PLoS ONE. 13: e0204234, 2018.

Bodó K, Kellermayer Z, László Z, Boros Á, Kokhanyuk B, Németh P, *et al*. Injury-induced innate immune response during segment regeneration of the earthworm, *Eisenia andrei*. Int. J. Mol Sci. 22: 1-23, 2021.

Boulias K, Greer EL. Means, mechanisms and consequences of adenine methylation in DNA. Nat. Rev. Genet. 23: 411-428, 2022.

Breiling A, Lyko F. Epigenetic regulatory functions of DNA modifications: 5-methylcytosine and beyond. Epigenetics Chromatin. 8: 24, 2015.

Brockes JP, Kumar A. Plasticity and reprogramming of differentiated cells in amphibian regeneration. Nat Rev. Mol Cell Biol. 3: 566-574, 2002.

Brockes JP, Kumar A. Comparative aspects of animal regeneration. Annu. Rev. Cell Dev. Biol. 24: 525-549, 2008.

Brockes JP, Kumar A, Velloso ACP. Regeneration as an evolutionary variable. J. Anat. 199: 3-11, 2001.

Çelik S. Understanding the complexity of antigen retrieval of DNA methylation for immunofluorescence-based measurement and an approach to challenge. J. Immun. Meth. 416: 1-16, 2015.

Chen C, Poss KD. Regeneration genetics. Annu. Rev. Genet. 51: 63–82, 2017.

Cooke JP. Inflammation and its role in regeneration and repair. Circul. Res. 124: 1166-1168, 2019.

Cooper EL. Earthworm immunity. Prog. Mol. Subcell. Biol. 15: 10-45, 1996.

Daponte V, Tylzanowski P, Forlino A. Appendage regeneration in vertebrates: what makes this possible? Cells. 10: 242, 2021.

Das PM, Ramachandran K, vanWert J, Singal R. Chromatin Immunoprecipitation assay. BioTechniques. 37: 961-969, 2004.

Duncan EM, Alvarado AS. Regulation of genomic output and (pluri)potency in regeneration. Ann. Rev. Gen. 53: 327-346, 2019.

Engelmann P, Hayashi Y, Bodó K, Ernszt D, Somogyi I, Steib A, *et al.* Phenotypic and functional characterization of earthworm coelomocyte subsets: linking light scatter-based cell typing and imaging of the sorted populations. Dev. Comp. Immunol. 65: 41-52, 2016.

Fernandes SB, Grova N, Roth S, Duca RC, Godderis L, Guebels P, *et al.* Methyladenine in eukaryotic DNA: tissue distribution, early embryo development, and neuronal toxicity. Front. Genet. 12: 657171, 2021.

Ferrario C, Sugni M, Somorjai IM, Ballarin L. Beyond adult stem cells: dedifferentiation as a unifying mechanism underlying regeneration in invertebrate deuterostomes. Front Cell Dev Biol. 8: 587320, 2020.

Galliot B, Crescenzi M, Jacinto A, Tajbakhsh S. Trends in tissue repair and regeneration. Development. 144: 357-364, 2017.

Gavin D, Sharma R, Chase K, Matrisciano F, Dong E, Guidotti A. Growth arrest and DNA-Damage-Inducible, beta (GADD45b)-mediated DNA demethylation in major psychosis. Neuropsychopharmacol. 37: 531–542, 2012.

Hendrich B, Tweedie S. The methyl-CpG binding domain and the evolving role of DNA methylation in animals. Trends in Genet. 19: 269-277, 2003.

Holstein TW. The Hydra stem cell system – Revisited. Cell Develop. 174: 203846, 2023.

Homa J, Zorska A, Wesoloski D, Chadzinska M. Dermal exposure to immunostimulants induces changes in activity and proliferation of coelomocytes of *Eisenia andrei*. J. Comp. Physiol. 183: 313-322, 2013.

Hostetter RK, Cooper EL. Earthworm coelomocyte Immunity. In: Cooper EL (ed.) Invertebrate immunology. Plenum Press New York & London, pp. 91-107, 1974.

Julier Z, Parker AJ, Briquez PS, Martino MM. Promoting tissue regeneration by modulating the immune system. Acta Biomaterial. 53: 13-28, 2017.

Kanherkar RR, Bhatia-Dey N, Csoka AB. Epigenetics across the human lifespan. Front. Cell Dev. Bio. 2: 49, 2014.

Katsuyama T, Paro R. Epigenetic reprogramming during tissue regeneration. FEBS Lett. 585: 1617-1624, 2011.

Kostyuchenko RP, Kozin VV. Comparative aspects of annelid regeneration: Towards understanding the mechanisms of regeneration. Genes. 12: 1148, 2021.

Kostyuchenko RP, Kozin VV. Morphallaxis versus epimorphosis? Cellular and molecular aspects of regeneration and asexual reproduction in annelids. Biol. Bull Russ. Acad. Sci. 47: 237-246, 2020.

Krenacs L, Krenacs T, Stelkovics E, Raffeld E. Heat-induced antigen retrieval for immunohistochemical reactions in routinely processed paraffin sections. Methods Mol. Biol. 588: 103-119, 2010.

Li Y, Tollefsbol TO. Combined chromatin immunoprecipitation and bisulfite methylation sequencing analysis. Methods Mol Bio. 791: 239-251, 2011.

Lunyak VV, Rosenfeld MG. Epigenetic regulation of stem cell fate. Hum. Mol. Genet. 17: 28-36, 2008.

Manzoni EF, Pennarossa G, de Eguileor M, Tettamanti G, Gandolfi F, Brevini TA. 5-azacytidine affects TET2 and histone transcription and reshapes morphology of human skin fibroblasts. Sci Rep. 6: 37017, 2016.

Mescher AL, Neff AW, King, MW. Inflammation and immunity in organ regeneration. Dev. Comp. Immunol. 66: 98-110, 2017.

Molnár L, Pollák E, Skopek Z, Gutt E, Kruk J, Morgan AJ, *et al*. Immune system participates in brain regeneration and restoration of reproduction in the earthworm *Dendrobaena veneta*. Dev. Comp. Immunol. 52: 269-279, 2015.

Morgan JM, Navabi H, Schmid KW, Jasani B. Possible role of tissue-bound calcium ions in citrate-mediated high-temperature antigen retrieval. J. Pathol. 174: 301-307, 1994.

Nix P, Hammarlund M, Hauth L, Lachnit M, Jorgensen EM, Bastiani M. Axon regeneration genes identified by RNAi screening in *C. elegans*. J Neurosci. 34: 629-645, 2014.

Özpolat BD, Bely AE. Developmental and molecular biology of annelid regeneration: a comparative review of recent studies. Curr. Opin. Genet. Dev. 40: 144-153, 2016.

Paul S, Balakrishnan S, Arumugaperumal A, Lathakumari A, Syamala SS, Vijayan V, *et al*. Importance of clitellar tissue in the regeneration

ability of earthworm *Eudrilus eugeniae*. Funct. Integr. Genomics. 22: 565-596, 2022.

Peiris TH, Hoyer KK, Oviedo NJ. Innate immune system and tissue regeneration in planarians: an area ripe for exploration. Semin Immunol. 26: 295-302, 2014.

Planques A, Kerner P, Ferry L, Grunau C, Gazave E, Vervoort M. DNA methylation atlas and machinery in the developing and regenerating annelid *Platynereis dumerilii*. BMC Biol. 19: 148, 2021.

Prochazkova P, Roubalova R, Dvorak J, Navarro Pacheco NI, Bilej M. Pattern recognition receptors in annelids. Dev Comp Immunol. 102: 103493, 2020.

Rasmussen KD, Helin K. Role of TET enzymes in DNA methylation, development, and cancer. Genes Dev. 30: 733-50, 2016.

Reddy PC, Gungi A, Unni M. Cellular and molecular mechanisms of *Hydra* regeneration. In: Tworzydlo W, Bilinski MS (eds) Evo-Devo: non-model species in cell and developmental biology. Springer, Heidelberg pp. 259-290, 2019.

Regev A, Lamb M, Eva J. The role of DNA methylation in invertebrates: developmental regulation or genome defense? Mol. Biol. Evol. 15: 880-891, 1998.

Rennolds CW, Bely AE. Integrative biology of injury in animals. Biol. Rev. Camb. Philos. Soc. 98: 34-62, 2023.

Sajadian SO, Ehnert S, Vakilian H, Koutsouraki E, Amm G, Seehofer D, *et a*l. Induction of active demethylation and 5hmC formation by 5-azacytidine is TET2 dependent and suggests new treatment strategies against hepatocellular carcinoma. Clin Epigenet. 7: 98, 2015.

Schikorski D, Cuvillier-Hot V, Leippe M, Boidin-Wichlacz C, Slomianny C, Macagno E, *et al*. Microbial challenge promotes the regenerative process of the injured central nervous system of the medicinal leech by inducing the synthesis of antimicrobial peptides in neurons and microglia. J. Immunol. 181: 1083-1095, 2008.

Shankland M, Seaver EC. Evolution of the bilaterian body plan: what have we learned from annelids? Proc. Natl. Acad. Sci. 97, 4434-4437, 2000.

Shao Y, Wang XB, Zhang JJ, Li ML, Wu SS, Ma XY, *et al*. Genome and single-cell RNA-sequencing of the earthworm *Eisenia andrei* identifies cellular mechanisms underlying regeneration. Nat Commun., 11: 2656, 2020.

Škanta F, Roubalová R, Dvořák J, Procházková P, Bilej M. Molecular cloning and expression of TLR in the *Eisenia andrei* earthworm. Dev Comp Immunol. 41(4): 694-702, 2013.

Stein EA, Cooper EL. Inflammatory responses in annelids. Am. Zool, 23: 145-156, 1983.

Tabish AM, Arif M, Song T, Elbeck Z, Becker RC, Knöll R, *et al*. Association of intronic DNA methylation and hydroxymethylation alterations in the epigenetic etiology of dilated cardiomyopathy. Am J Physiol Heart Circ Physiol. 317: 168-180, 2019.

Xiao N, Ge F, Edwards CA. The regeneration capacity of an earthworm, *Eisenia fetida*, concerning the site of amputation along the body. Acta Ecol. Sin. 31: 197-204, 2011.

Zheng P, Shao Q, Diao X, Li Z, Han Q. Expression of stem cell pluripotency factors during regeneration in the earthworm *Eisenia foetida.* Gene. 575: 58-65, 2016.