

Regeneration in stellate echinoderms: Crinoidea, Asteroidea, and Ophiuroidea

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1. A phylogenetic perspective of echinoderms

The phylum Echinodermata is divided in five extant classes: Crinoidea (sea lilies), Asteroidea (starfishes), Ophiuroidea (brittle stars), Holothuroidea (sea cucumbers) and Echinoidea (sea urchins). All classes are characterized by a rich fossil record (dating from the Cambrian period), which, in principle, should help to identify the relationships between the classes/species. However, this has not been the case and historically the classification of all classes has generated a heated debate. Recently, thanks to the introduction of large molecular datasets (mostly transcriptomic (Janies et al., 2016) and some genomic (Sodergreen et al., 2006; Hall et al., 2017), the field has undergone a profound transformation and has allowed different authors to reach a new consensus. Molecular phylogenomic studies clearly establish the Echinodermata phylum within the Deuterostomia, and a sister relationship with the Hemichordata (Ambulacraria = Echinodermata + Hemichordata; see: Cannon et al., 2014). Within the Echinodermata, Crinoidea (the only extant class of the Pelmatozoa subphylum) is the sister group of the remaining four classes, which form the subphylum of Eleutherozoa. Most recent phylogenies (O'Hara et al., 2014; Telford et al., 2014; Reich et al., 2015) support the existence of a clade that includes Asteroidea + Ophiuroidea (Asterozoa) and a clade with Echinoidea + Holothuroidea (Echinozoa) (Fig. 1A). This arrangement implies the existence of a common ancestor for all stellate echinoderms. An alternative hypothesis, not as well supported, places Ophiuroidea as sister to Echinozoa (Echinoidea + Holothuroidea). This hypothesis, known as the Cryptosyringida (Pisani et al., 2012), would support a different interpretation of adult and larval morphological evolution but will not be considered any further in this chapter.

The detailed combination of large, carefully selected, molecular and morphological characteristics further allowed the inference of intra-class relationships that were, until recently, difficult to trace (O'Hara et al., 2014; Thuy and Stöhr, 2016). These last investigations have led to specific propositions on the evolutionary changes associated with the diversification of each class and the reconstruction of ancestral states (Feuda and Smith, 2015), thus allowing the recognition of unique character changes occurring within each class (for instance characters linked to the regeneration process discussed in this chapter).

All extant classes of echinoderms (Candia Carnevali, 2006) as well as some species of hemichordates (Luttrell et al., 2016) are able to efficiently regenerate adult body parts. The fossil record largely supports the ancient origin of echinoderm regenerative potential, identifying regenerative structures in crinoids, asteroids and ophiuroids dating back to the Paleozoic era (Fig. 1 B and C; Oji, 2001). However, the extent and efficiency of regeneration can vary enormously

among echinoderms, a property that is linked to the ecological pressure created by predation (Oji, 2015).

Understanding the molecular underpinnings of ambulacrarian (or, specifically, echinoderm) regeneration will be critical for our understanding of the process in its varied presentations. Moreover, this knowledge should prove instrumental in deciphering how gene batteries and regulatory networks have changed over evolutionary time in order to meet the adaptation demands of each class (or constitutive clades). In this chapter we will review the studies undertaken to characterize cellular and molecular processes of regeneration in stellate echinoderms (Asterozoa + Crinoidea; Fig. 1A).

2. Echinoderm regeneration: not only a replacement

Regeneration is an intrinsically conservative post-embryonic developmental process providing not only an indispensable requisite for individual survival but also the necessary complement for the asexual reproduction programme. If a response to injury is evoked in all animals, the regeneration potential, *i.e.* the degree of morphological and functional recovery, actually varies significantly in different groups, in closely related species, and even in different tissues and organs of the same animal (Brockes and Kumar, 2008; Dinsmore, 2001; Sanchez-Alvarado and Tsonis, 2006; King and Newmark, 2012). The extent of the repair/regrowth is quite independent of the phylogenetic position of the group, depending exclusively on the individual histogenetic and morphogenetic potential that allows the expression of a new developmental program or the re-expression of the old one in the species.

In living echinoderms, the regenerative potential is expressed to a maximum extent and the regeneration processes, well described in old and recent literature, are frequent at all stages of the life cycle (embryo, larva, adult) and largely employed to rebuild body parts and even complete individuals from fragments following mutilation processes of different types (Candia Carnevali, 2006).

The extensive employment of regenerative potential throughout the phylum indicates that in echinoderms regeneration has the widest range of biological implications, increasing the chances of survival of the individual and, being the indispensable requisite for fissiparity, allowing the rapid colonization of new habitats through the production of multiple clones well adapted to local conditions. Hence, due to its evident adaptive and strategic significance/value, regeneration certainly contributes to the fitness of the species and to the success of echinoderms throughout the marine ecosystem.

Although in all echinoderm classes regenerative phenomena are extensively employed to reconstruct internal or external organs and structures (Candia Carnevali, 2006), arm regeneration in the stellate echinoderms represents one of the most useful and tractable experimental models for carrying comprehensive studies focused on ecological, developmental and evolutionary aspects as well as on involved mechanisms, cellular and molecular (Candia Carnevali, 2006; Dupont and Thorndyke, 2007; Franco et al., 2011a, b; Czarkwiani et al., 2016; Ben Khadra et al., 2017). In addition, in all the three groups the detached arm fragments (explants) can survive for a long time and undergo, as well, partial or total regeneration. In some asteroid species, a single arm can even regenerate a whole animal (Ben Khadra et al., 2017). The arm explants can therefore represent simplified and controlled *in vivo* regenerating systems, providing a valuable tool for testing mechanisms and processes (Candia Carnevali et al., 1998).

Although the ability to regenerate an arm occurs in all stellate echinoderms, differences in extent, timing, energetic costs and frequency of regeneration (Lawrence, 2010) are clearly observed within and among classes. These differences can be related to the specificities of the anatomy, physiology and behaviour of the species. In general, the long, thin and fragile arms of crinoids (Mladenov et al., 1983) and in faunal ophiuroids are frequently lost and usually regenerate more efficiently and rapidly than the larger asteroid arms (see next paragraph). For example, in natural populations of burrowing brittle stars (non-euryalid ophiuroids) up to 80-100% of individuals show signs of regeneration and have more than 70% of arms undergoing regeneration at one time (Wilkie, 1978; Bourgoin and Guillou, 1994; Clements et al., 1994; Sköld and Rosenberg, 1996; Soong et al., 1997; Yokoyama and Amaral, 2010). Regardless of the species, arm loss can occur for different reasons, including predation, environmental perturbation or intraspecific fighting (Emson and Wilkie, 1980; Ramsay et al., 2001).

In the different models explored so far, arms or explants, regeneration processes turned out to be an extremely complex development phenomenon that involves both repair and regenerative processes. They require not only new cell recruitment but also implementing detailed spatial information in order to properly specify the identity of the new tissues. Overall, the arm regeneration process tends to follow a common basic scheme, which is characteristic of the group and well understood in terms of costs, efficiency of regrowth and resources allocation. However, the same process can be, at the same time, plastic enough to achieve the same goal by changing the roles and contributions of the involved cells according to the individual situation (namely the mutilation preconditions but also age, health and nutrients availability) or the environmental conditions (Candia Carnevali, 2006). This means that each regeneration event is a very plastic phenomenon, which can be, and truly is,

intrinsically different for each individual or species, and can be adapted to the specific (environmental) requirements.

In the following paragraphs an up-to-date overview of the phenomenon of arm regeneration in stellate echinoderms is provided, and this is done by briefly revisiting and comparing the most thoroughly explored models, emphasizing differences and similarities, true or apparent, in the involved mechanisms at the tissue, cellular and molecular level. The comparison of results derived from different experimental arm models in the same phylum, employing different approaches, can provide insights on the specificity of processes, and their underlying regulatory mechanisms, governing large-scale pattern formation in newly formed tissues. Due to the complexity of the regenerative phenomenon it is obvious that comparing how different animal models achieve arm regeneration must be approached from multiple angles and requires the use of an integrate view.

3. Arm regeneration: the cellular and tissue perspective

The standard arm anatomy of crinoids, asteroids and ophiuroids has been reviewed by several classical works (Hyman, 1955; Harrison and Chia, 1994) and is here summarized in Fig. 2 for a generalized representative of each class. In this paragraph, we will recall only some of the main anatomical and architectural differences that have to be kept in mind in order to understand the observed diversity in regenerative mechanisms.

In general, it must be recalled that crinoids, being the only extant members of the Pelmatozoa sub-phylum, have an arrangement of anatomical structures opposite to that shown by the members of the Eleutherozoa (*i.e.* ophiuroids and asteroids), meaning that the oral side (the ambulacral groove) faces the water column rather than the substratum (as the latter group). They have three distinct nervous sub-systems (ectoneural, hyponeural and entoneural), which differ in anatomical localization and specific functions (Cobb, 1995). The most evident nerve structure of crinoid arm is the entoneural brachial nerve, which basically consists of a bundle of neurons and nerve processes running throughout the central part of the arm (Candia Carnevali et al., 1997). In both ophiuroids and asteroids, the main nervous structure is the radial nerve cord (RNC) that is mainly composed by a thick ectoneural neuroepithelium and a thin layer of hyponeural neurons and fibres (Hyman, 1955). In all the three classes the ectoneural system remarkably innervates both the outer (basi-epithelial plexus of the epidermis) and the inner epithelia (basi-epithelial plexus of the coelomic and visceral epithelia), giving rise to a diffused neural network reaching the main body organs (Harrison and Chia, 1994). Other evident inter-class differences are related to the diverse presence and arrangement of skeletal elements. Indeed, crinoids have very conspicuous columnar ossicles, which

occupy most of the arm and are serially repeated along it; the “armoured” ophiuroids have five distinct prominent elements (two lateral, an oral and an aboral shields and an inner conspicuous vertebra), again serially repeated along the arm with little connective tissue. The starfishes contain, instead, a meshwork of several medium sized ossicles immersed in abundant connective tissue giving to their body wall a “softer” consistency (Blowes et al., 2017).

3.1 Regenerative phases

The main histological events of the regenerative processes have been established in a series of studies following pseudo-autotomic or traumatic amputations (Mladenov et al., 1989; Candia Carnevali et al., 1993, 1995, 1997; Moss et al., 1998; Biressi et al., 2010; Ben Khadra et al., 2015a, b; Czarkwiani et al., 2016). In general, and regardless of the class, the overall process can be subdivided into three main phases: a **repair phase**, an **early regenerative phase** and an **advanced regenerative phase** (Fig. 3). These can differ in terms of timing and specific processes involved, both between classes and also within the same class. For example within ophiuroids, small size and frequently regenerating species, such as *Amphiura filiformis*, usually regenerate faster than large epibenthic species, such as *Ophioderma longicaudum* or *Ophioplocus januarii* (Biressi et al., 2010; Di Giorgio et al., 2015; Czarkwiani et al., 2016). In ophiuroids, different staging systems have been used (Biressi et al., 2010; Di Giorgio et al., 2015; Czarkwiani et al., 2016) (Fig. 4). In order to provide a generalized classification including all the different species studied so far and to allow an easier inter-class comparison with the other stellate echinoderms, we present here a reviewed staging system based on regenerative phases (Fig.5).

Regardless of the class and species, during the **repair phase** emergency reactions and wound closure must occur. First events are addressed to seal the main coelomic cavities, stop fluid loss and avoid the entrance of pathogens. In all the three classes this is ensured by very similar events, such as clotting phenomena by the free-wandering coelomocytes (which have also immune functions; Smith et al., 2010), rapid re-epithelialization by stretching and remodelling of the stump epidermal tissue and wound contraction/apical shrinkage to reduce the wound exposed surface (Candia Carnevali et al., 1993; Biressi et al., 2010; Ben Khadra et al., 2017; Ferrario et al., 2017). The latter event is particularly evident in asteroids, whereas it is negligible in crinoids, due to the intrinsic differences in their arm anatomy (vast somatocoel and relatively “soft” body wall of starfishes compared to the highly calcified arms of crinoids; see Fig. 2). Therefore, instead of contracting the whole arm wall, in the “armoured” arms of crinoids and ophiuroids, the muscle valves are present along the main axial canals/vessel (particularly the radial water canal), which ensure the sealing of

the canal itself upon arm injury and immediately stops the loss of body fluid (Candia Carnevali et al., 1993; Fig.2B in Czarkwiani et al., 2016). These valves are serially repeated along the arm, a property in line with the ability of these animals to perform autotomy at multiple (specific) arm levels and different to what occurs in starfishes, which have only one single autotomy plane at the base of the arm (Wilkie, 2001).

Re-epithelialization is rapid in all the three stellate echinoderms (in relation to their regeneration speed, Fig. 3) as it is accomplished at the middle of the repair phase (Candia Carnevali et al., 1993; Ben Khadra et al., 2015a; Ferrario et al., 2017). It is typically due to a stretching of the wound edge epidermal cells rather than to their proliferation (Candia Carnevali et al., 1995; Mladenov et al., 1989; Moss et al., 1998, Czarkwiani et al., 2016), this representing a major difference between echinoderms and mammals and possibly accounting for the more efficient repair (and therefore regenerative) ability of the former group (Ferrario et al., 2017).

In some asteroid species such as *Echinaster sepositus* a transient syncytium of phagocytes ensures stump tissue protection and provides a support for the overlying migration of epidermal cells (Ben Khadra et al., 2015a). A similar syncytial tissue has never been described in either crinoids or ophiuroids, although functionally comparable cell accumulations can be present (Ferrario et al., 2017). In large epibenthic ophiuroids, during the final step of the repair phase, a thick cicatricial layer is visible (Biressi et al., 2010; Di Giorgio et al., 2015); this is a tissue composed of the heterogeneous population of cells, including phagocytes, coelom-derived elements and apparently undifferentiated cells, immersed in a dense collagenous matrix. In many aspects, this layer histologically resembles the oedematous area (or granulation tissue) described in starfishes (Ben Khadra et al., 2015a, b, 2017), and therefore can be considered functionally and morphologically homologous. In starfishes and large ophiuroids, this structure possibly constitutes a “filling tissue” necessary in those large sized arms where the amputation plane following the trauma is concave and the first signs of regeneration appear after long time (Biressi et al., 2010; Di Giorgio et al., 2015). As in mammals (Pastar et al., 2014), this is a temporary tissue whose progressive maturation eventually leads to the formation of the scaffold for the subsequent regenerative growth. This event is generally not necessary for the thin arms of small brittle stars and crinoids, which complete the repair phase (and also the whole regenerative process) in just a few days (Fig. 5). Exceptions to this condition are those species living in extreme environmental conditions, such as the small Antarctic brittle stars *Ophiura crassa* and *Ophionotus victoriae*, where the repair phase is extremely delayed and the first signs of regrowth are visible only after several months (Clark et al., 2007; Clark and Souster, 2012). This delay, however, might be related to a generalized reduced

metabolic rate due to cold waters (Hughes et al., 2011) or to different energy allocation (Lawrence, 2010).

When the injury is completely healed, the **early regenerative phase** starts. In all the three classes of echinoderms these are the first genuine signs of cell differentiation, which will create the basis for the subsequent tissue regrowth. This includes the prominent event of axes determination (oral-aboral and proximal-distal). For those models where an epimorphic regenerative mechanism is employed, this phase is characterized by the appearance of a blastemal bud, a pool of undifferentiated cells which will, eventually, give rise to all the new arm structures (see next paragraph). A common feature shared by the three models is the concomitant regrowth of the main axial structures, which define the polarity along these axes: the coelomic cavities (both the perivisceral coelom and the radial water canal) and the nervous structures (the brachial nerve in crinoids and the RNC what is RNC? in ophiuroids and asteroids) with their associated sinuses. The regenerating tip of the coelomic cavities appears generally hypertrophic, probably as a result of a local and intense fluid secretion by the coelomic epithelia (Candia Carnevali et al., 1993; Biressi et al., 2010; Guatelli, 2017); this might have a functional consequences, leading to the generation of a hydrostatic pressure that allows the regrowth of the canals within the regenerate and also provides the turgidity and support to the regenerate itself, at least until the skeletal and muscle elements are completely developed. Therefore, use of the coelomic cavities as physical drivers of the regeneration process is a common principle used in stellate echinoderms that should not be neglected. Neural structures are also among the first tissues to be regenerated, and this is a feature that underlines their fundamental role played in echinoderm regeneration (see below). In the RNC of both ophiuroids and asteroids, the cell body layer of the neuroepithelium is first restored and it is only later that the neuropile, containing the nervous processes, becomes visible (Ben Khadra et al., 2015a, b; Czarkwiani et al., 2016). In the crinoids this differential regrowth is less evident, possibly due to the different anatomical organization of the brachial nerve (see above).

In asteroids and ophiuroids, the first signs of skeletogenesis also appear during this phase; this occurs particularly early in the former class and it might be related to the precocious development of the terminal ossicle, according to the so-called distalization-intercalation mode of regeneration (see below) (Czarkwiani et al., 2016; Ben Khadra et al., 2015b, 2017).

A fundamental aspect of the early regenerative phase that needs to be emphasized is the progressive re-population of the developing dermal (or “mesenchyme”) layer, located between the outgrowths of the axial structures and the regenerated epidermis, by relatively undifferentiated cells (Fig. 3). This is markedly evident in crinoids where this process ultimately gives rise to a typical blastema

(see next paragraph) (Candia Carnevali and Bonasoro, 2001). In starfishes, cell migration is largely occurring by the mobilization of different cytotypes from different areas, such as coelomic cavities and hyponeural sinus, all converging towards the regenerating area (Mladenov et al., 1989; Ben Khadra et al., 2015b).

Besides the onset of differentiation processes, during the early regenerative phase also dedifferentiation phenomena become clearly detectable, particularly in the muscular tissues including also those far from the amputation plane and not directly affected by the trauma (Candia Carnevali and Bonasoro, 2001; Biressi et al., 2010; Ben Khadra et al., 2017). Whether dedifferentiation is only a source of materials or also of cells remains an open question. Nevertheless, the concomitant occurrence of massive tissue (muscle) remodelling and tissue/structure regrowth (= increased metabolic needs) supports the idea that the remodelled tissue is acting as a source of energy/materials.

The **advanced regenerative phase** is characterized by the actual morphogenesis and final differentiation of all tissues, according to a proximal-distal direction of regrowth, a process involving tube feet, neural structures (*i.e.* the optic cushion in starfishes) and skeletal elements. Czarkwiani and co-workers (2016) noted that in *A. filiformis* the external shields (lateral, oral and aboral) differentiate earlier than the inner vertebrae suggesting a different regulatory developmental mechanism. Muscle bundles are generally the last tissues to be differentiated in line with their ontogenetic origin. They originate from the coelomic wall by myocyte ingression in the connective tissue situated below and migrate to their definitive site (Fig. 6; Rieger and Lombardi, 1987; García-Arrarás and Dolmatov, 2011).

For all echinoderm classes at the end of this stage the new miniaturized regenerate resembles the non-regenerating arm, in both morphology and functionality (Candia Carnevali and Bonasoro, 2001; Biressi et al., 2010; Ben Khadra et al., 2015b). In the starfish *E. sepositus* two tissues, namely pyloric caeca and papulae, show no signs of differentiation until four months post-amputation, possibly due to the fact that they are not fundamental for the small regenerate's immediate survival (Ben Khadra et al., 2015b). The regenerate, as the normal non-regenerating arm, will continue growing throughout the animal's life.

Regeneration rate can markedly vary among the three classes, and also within members of the same class. In general, crinoids and small ophiuroids display fast arm regeneration, whereas the big epibenthic ophiuroids and asteroids normally require longer periods for their complete arm regrowth. This might reflect size-related aspects or species/class-specific mechanisms of regeneration.

According to Dupont and Thorndyke (2006), in ophiuroids, depending on the amount of lost arm segments, the animal will invest more in the number of regenerated segments (and therefore in the overall regenerate length) or in the rapid differentiation of the regenerated segments. In other words, arms amputated more proximally (closer to the disc) will have longer but less differentiated regenerates, whereas those amputated more distally will rapidly differentiate the new segments but have shorter regenerates. Similarly, in asteroid, a proximally amputated arm regenerates a higher number of segments than a more distally amputated one over the same time period (over the same time-period; Sugni, Ferrario, Bonasoro and Candia Carnevali personal observations).

3.2 Blastema or not blastema?

Sanchez-Alvarado and Tsonis (2006) defined the regenerative blastema formed after the amputation as a structure “made of a superficial sheet of cells of epithelial origin covering the full extent of the bud, with an underlying localized mass of undifferentiated cells of mesenchymal origin”. These latter are typically characterized by a high proliferative activity and will later differentiate in all the specific lineages necessary to rebuild the lost body part. According to the classical definition by Morgan (1901) the regenerative mechanism is *epimorphic* when a localized blastema is formed, whereas it is *morphallactic* if no evident blastema is present and the cells involved in the regrowth come from the stump tissue after dedifferentiation, transdifferentiation and re-differentiation as well as migration of mature cells without intense and local proliferative activity (Candia Carnevali, 2006).

In this sense, arm regeneration in crinoids is a typical blastemal regeneration process in which new structures develop from a mass of pluripotent actively proliferating cells, which are responsible for both repair (Candia Carnevali et al., 1995) and tissue regeneration (Candia Carnevali et al., 1997). The blastema becomes clearly evident during the early regenerative phase (about three days post-amputation) as a pigment-less bud protruding from the amputation plane (inserts in Fig. 3). The blastema is mostly composed of morphologically undifferentiated cells, such as amoebocytes and coelomocytes. The former cells are normally located in the cortex of the brachial nerve, where they are in a “quiescent state” and can therefore be considered as a stem cell reservoir. Following the trauma they become active and migrate along the nerve towards the amputation plane (Candia Carnevali et al., 1995, 1997). The latter cells are morphologically very similar to the free-wandering cells of the coelomic fluid and are thought to derive from the coelomic epithelium, which they presumably cross to reach the underlying “mesenchymal” tissue that composes the blastema.

Starfish regeneration has been historically considered a “typical” morphallactic process, since a well-defined and localized blastema, *sensu stricto*, is never formed during the process, and cell

proliferation is widespread in different tissues, including those distant from the regenerating area (Bonasoro et al., 1998; Moss et al., 1998; Hernroth et al., 2010).

It is important to point out in this context that ophiuroid regeneration was traditionally considered an epimorphic blastemal process (Dupont and Thorndyke, 2006; Di Giorgio et al., 2015). However, a detailed re-examination of the available data and a deep inter-class comparison suggest that a true localized blastema (as that of crinoids) is never formed in these animals (Biressi et al., 2010; Czarkwiani et al., 2016) and that the regenerative mechanism is much more similar to that of asteroids than that of crinoids. As discussed above, the pre-blastemal area mentioned by Biressi and co-workers (2010) is similar to the oedematous area or granulation tissue of asteroids. Moreover, the true primordial regenerative bud (often improperly referred as the blastema) mainly contains the outgrowths of the coelomic cavities and RNC, which, although displaying a rather undifferentiated cell morphology and an intense proliferative activity at their distal-most end, always maintain their own histological (epithelial) individuality by being clearly delimited by their respective basal lamina (Czarkwiani et al., 2016; Ferrario et al., 2017; Piovani et al., 2017). In both the Eleutherozoan classes the area between the wound epithelium and the outgrowths of the axial structures, which in crinoids corresponds to the area of blastema formation, is never filled by a conspicuous localized mass of undifferentiated cells. What we detect is, instead, a zone of relatively loose mesenchyme in which, however, scattered and scarcely differentiated cells can be found immersed in a loose collagenous matrix (Fig. 3). This represents the so-called blastema-like structure described by Ben Khadra and co-workers (2017) in asteroids and the pools of mesenchymal/progenitor cells surrounding the tip of the coelomic canals and radial nerve cord mentioned by Biressi and co-workers (2010) and Piovani and co-workers (2017) in ophiuroids. Whether this is truly homologous to the crinoid blastema is an interesting issue to consider. Noteworthy, in all the three classes, most of the cells of this temporary tissue likely have, at least partially, a coelomic origin (Candia Carnevali et al., 1997; Ben Khadra et al., 2017; Piovani et al., 2017). In crinoids also the amoebocytes migrating from the brachial nerve contribute to the pool of blastemal cells (Candia Carnevali and Bonasoro, 2001). This might be related to the different organization of their nervous system, which is formed by bundles of fibres, perykaria and relatively “free” satellite cells (the amoebocytes) not constrained by a basal membrane, which is present in the RNC of the Eleutherozoans (Heinzeller and Welsch, 1994).

Overall, while the classification as blastemal/non blastemal regeneration can still be valid, the limitation of using the mutually “exclusive” terminology epimorphosis and morphallaxis is becoming more and more evident in echinoderms (Candia Carnevali, 2006) and, in general, animal regeneration (Agata et al., 2007). The coexistence of the two mechanisms, together with the

difficulties in the true understanding of the origin of cell composing the regenerate, make the use of this classification often ambiguous and inappropriate, and therefore, should be used with caution (Agata et al., 2007).

3.3 Regeneration-competent cells

Different types of cells are involved in echinoderm arm regeneration, some being specific of a class, others having inter-class roles. In crinoids the main players are migratory, morphologically undifferentiated, cells, such as amoebocytes and coelomocytes. These are hypothesized as pluripotent stem cells. Recent *in vitro* and *in vivo* studies allowed to identify the different progenitor cell types and to show their involvement in migration, proliferation, and dedifferentiation processes (Di Benedetto et al., 2014). In particular, *in vitro* results have confirmed the undifferentiated morphology of amoebocytes and their amoeboid activity. Indeed, as previously mentioned, during arm regeneration *in vivo*, these cells migrate along the brachial nerve towards the amputation site and undergo an extensive local proliferation in the developing blastema. Previous studies suggested that migratory amoebocytes ultimately produce both blastemal and blastema-derived differentiated cells. In contrast, the coelomocytes are supposed to give rise to all the differentiated elements related to coelomic tissues, including peritoneocytes, myocytes, and free coelomocytes, although a contribution to tissue re-growth of extra-coelomic compartments (*i.e.* skeletal tissue) cannot be excluded. Indeed, this would be consistent with previous studies (Czarkwiani et al., 2016; Piovani et al., 2017), which suggested that sclerocytes responsible for skeleton regeneration might originate from coelomic epithelium-derived progenitor cells.

In starfishes, cell recruitment has been traditionally considered to rely on stump cell dedifferentiation/transdifferentiation rather than on activation of stem cell stocks (Ben Khadra et al., 2017). However, Hernroth and co-workers (2010) recently proposed that cells of mixed origin might be recruited from distant sources of stem/progenitor pools rather than distant differentiated elements. This hypothesis was suggested based on the analyses of cell aging parameters (lipofuscin content, telomerase activity, etc.), which indicated how the cells composing the regenerate display “youth features” (typical of stem cells). Nevertheless, as suggested by the authors themselves, these characteristics might also be the result of a secondary rejuvenation process of already differentiated cells.

Besides pluripotent progenitor cells, in all the three classes of echinoderms, well-differentiated cytotypes also participate actively in the different phases of the regenerative process. These include phagocytes, specialized coelomocytes, granulocytes and pools of dedifferentiated cells, particularly myocytes (Candia Carnevali and Bonasoro, 2001; Candia Carnevali, 2006; Parma et al., 2006;

Candia Carnevali and Burighel, 2010; Di Benedetto et al., 2014). Phagocytes are the functional homologues of the vertebrate macrophages (Ottaviani, 2011): indeed, they are recruited and actively involved, particularly in the repair phase, when removal of cell debris and pathogens is necessary (Candia Carnevali et al., 1993; Biressi et al., 2010; Ben Khadra et al., 2017; Ferrario et al., 2017). As previously mentioned, in starfishes, these phagocytes can also form a transient syncytial structure covering the wound (Ben Khadra et al., 2017). Besides these specialized cells, the phagocytic activity is, however, a relatively common ability of all echinoderm cells, including epithelial cells (Sugni, Ferrario, Bonasoro and Candia Carnevali personal observation).

Because the “coelomocytes” include a very heterogeneous population of cytotypes (Smith et al., 2010) there is often a misunderstanding in their description and the roles they play. Coelomocytes *sensu stricto* should be referred to as the cells freely wandering in the coelomic fluid. As previously said, in crinoids these are morphologically undifferentiated cells that migrate and ultimately give rise to the blastema. In starfishes (as in other echinoderms, such as echinoids and holothuroids), these elements mostly include highly specialized and morphologically differentiated cells that are involved in clot formation, encapsulation and phagocytic activities as well as in the production of antibacterial compounds (Pinsino et al., 2007; Smith et al., 2010). The undifferentiated cells occasionally observed within the coelomocyte population are considered to be a progenitor source of these highly differentiated cytotypes (Gorshkov et al., 2009; Guatelli, 2017). Therefore, in starfishes the *sensu stricto* coelomocytes are mainly involved in the reparative events and in the first immune response following the injury (Pinsino et al., 2007; Gorshkov et al., 2009; Holm et al., 2008; Ramírez-Gómez and García-Araráz, 2010; Sharlaimova et al., 2010; Sharlaimova and Petukhova, 2012). Coelothelium-derived cells, instead, likely contribute to new tissue regrowth (see paragraph 3.5)

Other types of migratory cells involved in regeneration include the granulocytes or “wanderzellen” (Reichensperger, 1912) of crinoids. These are randomly scattered in all tissues, although particularly localized around the brachial nerve and in the regenerating tissues during the first regenerative phases. It has been hypothesized that these cells could be a source of putative growth factors even if, so far, they have not been identified (Thorndyke and Candia Carnevali, 2001).

A remarkable common feature of echinoderm arm regeneration (and generally of all echinoderm regenerative processes) is the “recycling” and plasticity of adult stump cells. This occurs particularly in muscle tissues (muscle bundles as well as coelom-associated myocytes), and, to a minor extent, in other mesodermal cell types (*i.e.* skeleton, ligaments, etc.; Candia Carnevali et al., 1993; Candia Carnevali and Bonasoro, 2001; Candia Carnevali, 2006; Czarkwiani et al., 2016; Ben Khadra et al., 2017). From a histological point of view this is evidenced by myocytes losing their

typical fusiform shape and showing signs of disorganization in their contractile apparatus, which is progressively compacted. At present it is still unclear the real fate of these remodelled myocytes and their physiological significance for the regenerative process. Two hypotheses can be considered (Fig. 7): 1) myocytes undergo a true dedifferentiation process by extrusion of their contractile apparatuses and production of characteristic spindle-like structures (SLS; García-Arrarás and Dolmatov, 2011) (eventually used as source of energy/material) while the remaining cell body becomes a regeneration-competent pluripotent cells; 2) they undergo nuclei degeneration (apoptosis) and the remaining SLS are therefore used just as a source of material/energy for the regenerative process (Ben Khadra et al., 2017). Specific cell tracking or the use of molecular tools will hopefully help to clarify this aspect. Nevertheless, it is interesting to stress that in the remodelling muscles no conspicuous signs of apoptosis (*i.e.* nuclei with abnormally condensed chromatin) have generally been noticed at the Transmission Electron Microscope (TEM) level (Sugni, Ferrario, Bonasoro and Candia Carnevali personal observations).

In the present chapter the remodelling phenomena of injured tissues (*i.e.* directly involved in the amputation), which normally undergo histolytic processes, are not considered.

In regeneration muscle remodelling is remarkably relevant in asteroids (Ben Khadra et al., 2017). By contrast, in ophiuroids and crinoids this phenomenon is generally of minor importance and usually only a limited cell turnover can be observed at the periphery of the muscle bundles (Candia Carnevali and Bonasoro, 2001; Biressi et al., 2010). In crinoids, however, myocyte remodelling remarkably increases under stress conditions, such as following exposure to contaminants or in arm explants (Bonasoro et al., 1998; Candia Carnevali et al., 2001a, b, 2003; Sugni et al., 2007). In all the three echinoderm classes, muscle remodelling often occurs *via* a more or less evident distal-proximal gradient (*i.e.* muscle bundles or tube feet muscle layers closer to the amputation plane are the most remodelled; Ben Khadra et al., 2015a). This fact might indicate the presence of a gradient of triggering molecules “released” at the wound site (such as inflammatory-like molecules) that diffuse along the arm.

The phenomena of tissue rearrangement can involve also the extracellular matrix and other cytotypes, *i.e.* fibroblasts, scleroblasts, cells of the coelomic epithelium or, in asteroid arm explants, even cells of the digestive apparatus (*i.e.* pyloric caeca) (Ben Khadra et al., 2015a, 2017), thus highlighting the plasticity of echinoderm tissues and the ability of these animals to use different sources of materials or cells according to the specific conditions and needs. However, as for myocytes, the final fate of these dedifferentiating cells remains unclear. The absence of specific cell tracking systems makes impossible to definitively demonstrate if they are actually employed as a secondary indirect source of raw materials for the production of new cells or as primary direct

sources of undifferentiated/pluripotent cells (or perhaps, a combination of both). Regardless of this conundrum, the uncommon plasticity and “recycling” ability of echinoderm adult cells are certainly one of the “secrets” of their amazing regenerative abilities with the possible existence of “cell rejuvenation processes” in these animals opening fascinating perspectives for translation to the fields of aging and human regeneration.

3.4 Distalization-intercalary regeneration

To overcome the ambiguity and difficulties of classifying a regenerative process as epimorphic or morphallactic, in 2003 Agata and co-workers (Agata et al., 2003, 2007) proposed a different but unifying principle to approach regeneration in different animal models, such as planarians, cnidarians (*Hydra*) and amphibian urodeles. This view was articulated as the distalization-intercalary regeneration model. According to this model, the most distal structure is formed first and this acts as a signalling centre for organising and inducing the intercalation of new elements between the stump and the distal element itself. This model has been recently successfully applied also to echinoderm arm regeneration (Fig. 8; Hotchkiss, 2009; Ben Khadra et al., 2015b, 2017; Czarkwiani et al., 2016), although differences in the modelled structures are obvious in the three classes. For example, in asteroids, the distal element is the terminal tube foot with its associated ossicle, which are highly differentiated structures. In crinoids this is actually the blastema itself, as it is the case in planarians (Agata et al., 2003), which is an undifferentiated tissue. Ophiuroids represent a peculiar situation since the terminal structures (*i.e.* terminal tube foot and associated ossicle) are morphologically well recognizable only at late stages of regeneration but once differentiated they likely act as the asteroid’s counterpart (Czarkwiani et al., 2016). In both classes the actual growth zone is therefore located proximal to the terminal structure (Ben Khadra et al., 2015b, Czarkwiani et al., 2016).

An alternative and unifying hypothesis is that the true distal organizer in all three echinoderm classes is the anatomical region (not specifically definable) where the tips of the axial structures (*i.e.* coelomic cavities and nerve) meet, which is actually the growth zone proper. This might be the real key area and a structure controlling the whole arm regeneration process, where the new structures originate.

Noteworthy, in the animal models used by Agata and co-workers (2003) the distal element is usually a transient structure that disappears once regeneration is accomplished. Differently, in echinoderms, the distal organizer is a permanent structure that remains active throughout life, in agreement with the continuous arm growth. In this sense echinoderm regeneration can be seen as a

regulative phenomenon, which reproduces, in accelerated form, the usually slow but continuous growth of the arms.

Some exceptions to this model can be found in specific conditions, as in crinoid unconventional amputations (*i.e.* not on the natural autotomy site; Candia Carnevali and Bonasoro, 1995) or in asteroid arm explants (Ben Khadra et al., 2017). In the former, a delayed repair phase, due to an amputation far from the natural arm autotomic plane or due to a basal arm mutilation and subsequent exceptional extent of the wound, may produce abnormal regenerates. In this case, only the distal part is well differentiated, whereas the intermediate part presents evident anomalies (Candia Carnevali and Bonasoro, 1995). In asteroid double arm explants (*i.e.* the intermediate part of an arm originated from a proximal and a distal cut) the distal side regenerates following the distalization-intercalary model (although much delayed if compared to normal arm-tip regeneration). In contrast, at the proximal side only distalization is observed *i.e.* only the distal element, the terminal tube foot, is regenerated but no new segment is intercalated between the latter and the stump (Valoti et al., 2016).

3.5 Coelom and nervous tissue as the key players of echinoderm regeneration

3.5.1 The coelomic epithelium as organogenetic tissue

The coelomic epithelium (also referred to as “peritoneum”) is considered a unique tissue for its histogenetic potential (Rieger e Lombardi, 1987). In vertebrate embryogenesis this is a highly active cell layer, which is locally able to supply mesenchymal cells that contribute to the mesodermal elements of many organs, such as heart, liver, lungs, gonads and more, and provide essential signals necessary for the development of other cells, tissues and organs (Ariza et al., 2016). From a cellular point of view, cell recruitment occurs by epithelial-mesenchymal transition (EMT). During this process, the epithelial cells lose their polarity as well as their cell-junctions and migrate into the underlying connective tissue (mesenchyme) to become mesenchymal cells, *i.e.* “free” undifferentiated or multipotent cells. The EMT is a key event during both echinoderm embryo development (Wu et al., 2007) and adult regeneration (Ben Khadra et al., 2017; Guatelli, 2017). Indeed, the coelomic epithelium has been proposed to act as an “ancient multifunctional organogenetic tissue” also in the regenerative development of different echinoderm classes (Dolmatov, 1993; Candia Carnevali et al., 1995; García-Arrarás et al., 2011). A significant role of the coelomic structures was originally suggested by pilot studies carried out during the last century on ophiuroids (Dawydoff, 1901). In recent times this idea was re-proposed for all the stellate echinoderms (Candia Carnevali and Bonasoro, 2001; Bannister et al., 2008; Biressi et al., 2010; Ben Khadra et al., 2017; Piovani et al., 2017). Most of these studies indicate that in echinoderms the coelom is a fundamental cell supplier during regeneration (see the paragraph 3.3) with the

somatocoel being apparently the primary source of cells in all the three classes (Candia Carnevali et al., 1993; Biressi et al., 2010; Ben Khadra et al., 2017), although a contribution of the radial water canal has also been suggested (Bannister et al., 2008). Although cell tracking experiments are still necessary, in both asteroids (Ben Khadra et al., 2017; Guatelli, 2017) and ophiuroids (Piovani et al., 2017) EMT has been proposed as a specific mechanism recruiting cells from the coelomic epithelium to the surrounding mesenchymal tissue, similar to what has been described in holothurian visceral regeneration (García-Arrarás et al., 2011) and vertebrate embryogenesis (Ariza et al., 2016). Thus, it is reasonable to consider the adult echinoderm coelomic epithelium as an extremely plastic tissue, which is highly specialized and differentiated in normal physiological conditions but that can be turned into a “multipotent cell supplier” upon the reception of specific signals and under stress conditions, such as those generated by traumatic amputation.

3.5.2 Nervous system as coordinator

The key role of the nervous system during the regenerative process is well documented in all three echinoderm classes (Huet, 1975; Huet and Franquinet, 1981; Walsh et al., 1986; Moss and Thorndyke, 1994; Moss et al., 1998; Candia Carnevali et al., 2001c; Thorndyke and Candia Carnevali, 2001; Sugni et al., 2010) and other animal models (Dinsmore and Mescher, 1998; Tanaka and Ferretti, 2010; Kumar and Brockes, 2012). In echinoderms, the nervous system acts as a promoter/coordinator of the overall regenerative process, mainly contributing to the release of regulatory factors. These factors have been identified by both qualitative and quantitative methods (immunocytochemistry and histofluorescence, biochemical assays and molecular techniques), which have revealed the presence of at least three different classes of regulatory molecules involved to some extent in regeneration: neurotransmitters, particularly mono-amines, such as dopamine and serotonin; neuropeptides, such as Substance-P, SALMFamide 1 (S1), SALMFamide2 (S2); or nerve-derived growth factors, such as TGF- β and related peptides (BMP), NGF, FGF-2 (Candia Carnevali et al., 1996; Thorndyke and Candia Carnevali, 2001; Patruno et al., 2002, 2003).

In asteroids, the “exposed” anatomical position of the RNC, which is facing the external environment (Fig. 2; Smith, 1937), allows to perform ablation experiments *i.e.* partial removal of the nerve cord. When this has been done during arm-tip regeneration experiments the regrowth process of the arm-tip has been markedly impaired, although not completely prevented (Moss et al., 1998). In crinoids, the “silencing” of nervous activity by pharmacological treatment causes a decrease in the proliferative activity of the regenerate, which in turn ultimately results in a reduced regrowth (Sugni et al., 2010). Although the specific molecular pathways involved are still unknown, these different evidences underline the shared nerve-dependence of arm regeneration in the different echinoderm classes.

It is important to recall that the nervous system includes not only the anatomically well-defined component of the brachial nerve but also the diffuse conspicuous components of the basic epithelial plexuses, particularly the sub-coelothelial plexus associated to the coelom (Harrison and Chia, 1994). This means that the basic “pilot-role” played by the nervous system in regeneration would really imply a significant contribution from the coelomic plexus. In other words, the prompt and effective contribution of coelomic cells that can be observed in all the echinoderm regeneration models studied so far (see above), in terms of migration, proliferation and differentiation, could be a phenomenon regulated by the local release of neural factors from the different nerve components. In crinoids the nervous system seems also to exert a specific direct induction, as far as the organization/differentiation of other tissues is concerned, an effect that is particularly evident in the muscle-skeletal components (Candia Carnevali et al., 1989, 1999).

In summary, in the paragraphs above we discussed how stellate echinoderms display an extraordinary variety of regenerative mechanisms, in terms of cells and the tissues involved. This diversity (Table 1) can be ascribed to diverse factors, including evolutionary, ecological and individual (physiological) aspects and thus they can provide a valuable perspective on how the same process (regeneration) can be performed by different routes.

Table 1 Summary table of the main differences among the stellate echinoderm regenerative process described in the previous paragraphs, focusing on a cell/tissue perspective.

Event/mechanism/process	Crinoids	Ophiuroids	Asteroids
Transient phagocyte syncytium			x
Granulation tissue-like formation		x	x
Presence of a true localized blastema	x		
Terminal tube foot and ossicle as distal structures		x	x
Differential regeneration of nervous system components		x	x
Maintained regeneration polarity in arm explants	x	x	
Stem cell recruitment	massive	reduced	reduced
Adult cell dedifferentiation	reduced	massive	massive
Prevalent regenerative mechanism	epimorphosis	morphallaxis	morphallaxis

4. Arm regeneration: the molecular perspective

Though the basic morphological aspects of echinoderm regeneration are relatively well known (see the above sections) the paucity of data on the regulatory mechanisms involved in this developmental process is hampering our progress, underlying the necessity of improving our analysis of the molecular aspects of the process and the incorporation of new technologies that allow us to interfere with it. In the next sections we describe the knowledge we have gathered over the last years on this subject.

4.1 Crinoids

In spite of the striking regenerative potential that crinoids display, most of the analyses on this process have been done at the cellular level and the molecular studies are still limited. The first molecular study on crinoid regeneration was carried out in 1996 and aimed at investigating the potential role of the nervous system in regeneration by testing the specific involvement of neural factors (the monoamine neurotransmitters dopamine and serotonin) in arm regeneration of *Antedon mediterranea* (Candia Carnevali et al., 1996). The distribution pattern of these molecules at standard regenerative stages was compared with that of normal non-regenerating arms. Immunocytochemical (ICC) examination showed that both dopamine and serotonin dramatically change in both their distribution and concentration during the repair and regenerative processes, indicating that they could be important neural (perhaps growth-promoting) factors used in crinoid arm regeneration (Candia Carnevali et al., 1996). Other than that, the control and the initiation of the regenerative response are not mediated only by neurotransmitters. Other different regulatory molecules are probably involved. So far, only one family of factors native to crinoids has been identified in regenerating tissues: the members of the transforming growth factor- β (TGF- β) group of signalling molecules. Secreted proteins of the TGF- β superfamily are dimeric cytokines involved in fundamental aspects of wound healing and regeneration invertebrates (Lévesque et al., 2007). The expression of TGF- β -like molecule(s), detected using an anti-human TGF- β 1 antibody, is significantly up-regulated at the level of the regenerating brachial nerve in the apical blastema of *A. mediterranea* (Patrino et al., 2002). In the early stages of regeneration, localization of TGF- β 1 is detected in several migratory amoebocytes close to the amputation surface, whereas the TGF- β type II receptor is detected in migrating amoebocytes in the brachial nerve (Patrino et al., 2002). These data indicate that a TGF- β -like molecule (or molecules) is normally present in the adult nervous tissues of *A. mediterranea* and is significantly up-regulated during regeneration. Later on, Patrino and co-workers (2003) reported the sequence of another identified native member of TGF- β superfamily from *Antedon bifida*, belonging to the bone morphogenetic proteins (BMPs) subfamily (named: *Anbmp2/4*). BMPs are a group of multifunctional growth factors involved in embryonic and regenerative development (Chen et al., 2004). They demonstrated that *Anbmp2/4* is actively involved in the early stages of blastemal regeneration, at a time when fundamental patterns are being established. The authors speculated that *Anbmp2/4* could be involved in the specification of migratory stem cells derived from the coelomic epithelium (Patrino et al., 2003).

To check the origin of stem cells, different “stemness” (or stem-related) genes, such as *vasa* and *piwi* (*unpublished*), plus some regulatory genes involved in anterior-posterior patterning of embryos, for example *otx* and *pax6*, were identified in *Oxycomanthus japonicus* embryos and adult

tissues (Kondo and Akasaka, 2010). The detailed analysis of these genes during regeneration may clarify the identity and fate of stem cells in these animals. Moreover, and in order to promote studies of crinoids at the molecular level, this research group constructed cDNA libraries of embryos and adult tissues from *O. japonicas* cultures, which bring the system to the state of becoming a “model” for studying regeneration in crinoids, as it is in the previously described *A. mediterranea*.

Unfortunately, to date, only these above-mentioned molecules have been investigated in crinoid regeneration. This fact stresses the urgency of performing molecular studies on the regulatory mechanisms that control regenerative processes in this class of echinoderms.

4.2 Asteroids

Although different studies have been carried out with the aim of determining the detailed morphological aspects of asteroid regeneration (reviewed in Ben Khadra et al., 2017), there is still, as stated, a considerable gap in the knowledge of the molecular aspects regulating the process. Some studies were conducted attempting the identification and cloning of some genes involved in regeneration. These molecules were mainly identified in the context of asteroid larval regeneration. The first characterised molecule was the so-called “asteroid regeneration-associated protease” (*srap*) in the asteroid *Luidia foliolata* during its larval regeneration (Vickery et al., 2001). Following a similar approach, Ouhlen and co-workers (2016) identified a *srap* gene and two other genes, *vasa* and *vitellogenin* relatives, involved in the regeneration of *Patiria miniata* bipinnaria larvae. These approaches did not involve an exhaustive characterisation of the gene function; however, the authors speculated that the *srap* gene might have a role in wound repair and apoptosis. Some other studies aimed at the characterization of a small set of regulatory molecules/genes involved in adult asteroid regeneration. These studies characterized some members of families with known roles in regulating key aspects of embryonic and post-embryonic development, such as *homeobox*-containing and *wingless* genes. Ben Khadra and co-workers (2014) used a PCR screening protocol to identify different *Hox* and *ParaHox* genes putatively involved in arm regeneration of *E. sepositus* and *A. rubens*. Based on comparisons with vertebrate and *Strongylocentrotus purpuratus* *Hox/Hox*-related fragments and with the use of phylogenetic analysis, eight distinct *Hox* genes expressed in regenerating *E. sepositus* and six in regenerating *A. rubens* were characterized, in addition to four putative homologs of the vertebrate genes *Xlox* in *E. sepositus*, *Mox* in *A. rubens*, and *Gbx* in both species. All *Hox* fragments showed the clearest affinity with deuterostome genes (the so-called anterior, medial, and posterior groups) (Ben Khadra et al., 2014). Among all the characterized *Hox* and *ParaHox* genes identified by Ben Khadra and

co-workers (2014), only the expression of *Hox1* has been analysed during asteroid regeneration by using a species-specific antibody. Thorndyke and co-workers (2001) showed that *Hox1* is expressed at low levels in normal radial nerve cord of *A. rubens* and then it is up-regulated during its regeneration. The authors speculated that the expression of *Hox1* seen in whole-mount ICC could interfere with the dedifferentiation and/or cell proliferation process during regeneration (a hypothesis never formally investigated).

Ben Khadra and co-workers (*unpublished*) characterized members of the *wingless/Wnt* family genes present in regenerating tissue of *E. sepositus*. *Wnts* are a family of secreted glycoproteins with significant roles in many biological processes. Beside the crucial role of *Wnt* genes during early embryonic developmental processes, it has been demonstrated that *Wnt* signalling is also required for adult tissue regeneration in different species (Wodarz and Nusse, 1998; Niehrs, 2001; Veeman et al., 2003). Ben Khadra and co-workers identified specifically two *Wnt* genes expressed in the *E. sepositus* regenerating arms, the orthologous of *Wnt1* and *Wnt4* genes, named here respectively *EsWnt1* and *EsWnt4*. The expression profiles of both genes at different stages of this starfish arm regeneration were studied by RT-PCR. The expression profiles of *EsWnt1* and *EsWnt4* genes showed a variation in different stages of regeneration, with the highest expression detected at 24 and at 72 hours post-amputation. These findings suggest that these two genes are probably involved in the wound healing process. In fact, it is known that the activation of the *Wnt* signalling pathway is an early response to trauma in different species (Whyte et al., 2012). In starfishes this initial phase is followed by a late expression at 4 and 7 weeks post-amputation where the expression of *EsWnt1* and *EsWnt4* coincides with the morphogenesis of some missing tissues, such as skeletal elements and muscles. These preliminary investigations need further experiments to validate the specific roles played by each gene in specific processes of *E. sepositus* regeneration.

In a recent study (Ferrario et al., 2017), the expression patterns of two genes were described during the repair phase of the asteroid *E. sepositus*. A fibrinogen-like gene (*Ese-fib-like*) was detected in the regenerating epidermis and coelomic epithelium, suggesting an involvement of these tissues in the post-traumatic inflammatory response. Indeed, fibrinogen is a well-known molecule involved in vertebrate haemostasis following injury (Laurens et al., 2006). The expression of a prolyl-4-hydroxylase gene (*Ese-p4h*), a key enzyme for collagen biosynthesis, in the regenerating epidermis suggested that this tissue is involved in collagen deposition during the repair phase, when the oedematous area is formed (Ben Khadra et al., 2017).

An alternative study of the regenerative process has been done in asteroids using proteomic tools. By means of these technologies, *Wnt* (and other) proteins belonging to this signalling pathway were identified in the regenerates. This finding is in line with the suggestion that *Wnt* signalling

controls stem cell operation and provides the positional information necessary for shaping the regenerating tissues (Clevers et al., 2014). Proteins of this pathway have been clearly identified in the regenerating RNC on the starfish *M. glacialis* (Franco et al., 2011a). A preliminary study performed on this species showed significant differences between the coelomocytes' proteomes in regenerating animals and those of the wild type animals (*unpublished data*). This finding suggests that the coelomocytes are key participants of the regenerative process in line with what has been observed at the cellular level. Indeed, the proteome characterization of coelomocytes during regeneration in starfishes showed the up and down regulation of the Heat shock proteins (Hsp), which are key regulators of cell homeostasis. In a different species, *A. rubens*, an increase in Hsp72 levels was observed in coelomocytes after 24 h post amputation (Holm et al., 2008b) and in the RNC until 6 weeks post-amputation (when compared to the control, non-amputated, specimens, that showed no changes of Hsp70 levels (Patrino et al., 2001)). Several other proteins are specifically involved in neurogenesis during regeneration. For instance, a total of 528 proteins were detected having significant variations in their expression during starfish neural regeneration in *in vivo* using difference gel electrophoresis (2D-DIGE) (Franco et al., 2014). The authors suggested that these molecules might modulate the regenerative pathways leading to successful nervous system regeneration in the starfish (reviewed in Ben Khadra et al. 2017). It is important that proteomic characterisation of the RNC of an asteroid model revealed a close similarity between echinoderm and mammal central nervous system physiology, in particular their synaptic communication components (Franco et al., 2011a). A more extensive analysis is needed in order to understand the basic mechanisms that regulate the regeneration of asteroid tissues and how these different proteins act during this process. The future use of similar technologies in members of other asteroid clades should provide us with insights on the commonalities and differences between the regenerative processes in the Asteroidea and beyond.

In parallel to the mentioned proteomic studies, other global (high-throughput) approaches, using the analysis of transcriptomes and/or genomes, are starting to be used. These approaches give us an unprecedented view at biological processes, such as regeneration, and how their regulation is effected at the transcriptional level in other asteroids (Gabre et al., 2015; Semmens et al., 2016; Hall et al., 2017).

Even though starfish regeneration started to attract researchers' attention long time ago, there is still a big gap which needs to be urgently filled in order to understand how the independently gathered cellular and the molecular data fit together.

4.3. Ophiuroids

Among the stellate classes, the Ophiuroidea are the best studied at molecular level for their ecological and regenerative importance. In recent years, with the rising of easy and low-cost next generation sequencing (NGS) approaches, there has been an emergence of various molecular resources and genome projects (summarized in Table 2) that greatly helped and will continue to help, filling the gap of molecular mechanisms underlying echinoderm regeneration. Several studies have been done on single gene expression and large gene expression data sets of ophiuroids, including transcriptomic and proteomic approaches; however, here we will focus only on those relevant to the regenerative processes.

Table 2. Ophiuroidea genomic and transcriptomic resources publicly available.

Species	Genome (G)/Transcriptome (T)	NCBI	URL
<i>Ophiotrix spiculata</i>	G	PRJNA182997	http://www.echinobase.org/Echinobase/OsAbout
<i>Astrophyton muricatum</i>	Adult body T	PRJNA299886	http://echinodb.uncc.edu/
<i>Ophioderma brevispina</i>	Adult body T	PRJNA299887	http://echinodb.uncc.edu/
<i>Amphiura filiformis</i>	Embryo and Adult T	PRJNA349786; PRJNA256912; PRJNA256029; PRJNA244369; PRJNA192896	http://www.echinonet.eu/blast/
<i>Ophiorensis fascinata</i>	G	GCA_900067615.1	http://ryanlab.whitney.ufl.edu/genomes/Ofas/
<i>Ophionotus victoriae</i>	Adult body T	PRJNA273557; PRJNA387478	British Antarctic Survey
<i>Ophioderma longicaudum</i>	Adult body T	PRJNA360063	University of Basel
<i>Ophiopsila aranea</i>	Adult body T	PRJNA266533	University of Mons
<i>Ophiolimna perfida</i>	Adult body transcriptome	SRX1625120	Museum Victoria
<i>Ophiocoma wendtii</i>	Adult body transcriptome	SRX1625119 & PRJNA299897	Museum Victoria
<i>Ophioleuce brevispinum</i>	Adult body transcriptome	SRX1625118	Museum Victoria
<i>Ophiomusium lymani</i>	Adult body transcriptome	SRX1625117	Museum Victoria
<i>Ophioceres bispinosus</i>	Adult body transcriptome	SRX1625116	Museum Victoria
<i>Amphiophiura laudata</i>	Adult body transcriptome	SRX1625115	Museum Victoria
<i>Ophiactis resiliens</i>	Adult body transcriptome	SRX1625114	Museum Victoria
<i>Ophiophragmus wurdemanii</i>	Adult body transcriptome	SRX1625113	Museum Victoria

<i>Ophiothrix caespitosa</i>	Adult body transcriptome	SRX1625112	Museum Victoria
<i>Ophiopeza cylindrica</i>	Adult body transcriptome	SRX1625111	Museum Victoria
<i>Ophiophrura liodisca</i>	Adult body transcriptome	SRX1625110	Museum Victoria
<i>Ophiophycis johni</i>	Adult body transcriptome	SRX1625109	Museum Victoria
<i>Ophiologimus prolifer</i>	Adult body transcriptome	SRX1625108	Museum Victoria
<i>Gorgonocephalus pustulatum</i>	Adult body transcriptome	SRX1625107	Museum Victoria
<i>Ophiolepis impressa</i>	Adult body transcriptome	SRX1625106	Museum Victoria
<i>Clarkcoma canaliculata</i>	Adult body transcriptome	SRX1625105	Museum Victoria
<i>Ophiura sp. MVF193435</i>	Adult body transcriptome	SRX1625104	Museum Victoria
<i>Bathypectinura heros</i>	Adult body transcriptome	SRX1625103	Museum Victoria
<i>Ophiernus vallincola</i>	Adult body transcriptome	SRX1625102	Museum Victoria
<i>Ophiotreta eximia</i>	Adult body transcriptome	SRX1625101	Museum Victoria
<i>Ophiomoeris obstricta</i>	Adult body transcriptome	SRX1625100	Museum Victoria
<i>Asteroschema bidwillae</i>	Adult body transcriptome	SRX1625099	Museum Victoria
<i>Ophiomyxa australis</i>	Adult body transcriptome	SRX1625098	Museum Victoria
<i>Asteronyx loveni</i>	Adult body transcriptome	SRX1625097	Museum Victoria
<i>Ophionereis schayeri</i>	Adult body transcriptome	SRX1625096	Museum Victoria
<i>Amphiura constricta</i>	Adult body transcriptome	SRX1625095	Museum Victoria
<i>Ophiactis abyssicola</i>	Adult body transcriptome	SRX1625094	Museum Victoria
<i>Leptosynapta tenuis</i>	Adult body transcriptome	SRX1625093	Museum Victoria
<i>Ophiothrix angulata</i>	Adult body transcriptome	SRX1625092	Museum Victoria
<i>Aporometra wilsoni</i>	Adult body transcriptome	SRX1625091	Museum Victoria
<i>Luidia senegalensis</i>	Adult body transcriptome	SRX1625090	Museum Victoria

Genes encoding two proteins of the family of bone morphogenetic factors (BMPs) have been isolated and studied in the context of *A. filiformis* arm regeneration. *AfUni*, closely related to the sea urchin *Univin* gene, and *AfiBmp2/4* have their expression detectable only in advanced regenerative phase (3 weeks and 2 weeks post-amputation respectively) in cells with a migratory morphology

within the radial water canal(RWC) of the regenerates (Bannister et al., 2005, 2008). The authors have interpreted this as a supporting evidence for the RWC as being an important source of cells in *A. filiformis* regeneration. Furthermore, *AfiUni* expression is localized in two regions of the regenerates: a proximal region and the most distal tip. This led the authors to suggest a dual role of *AfiUni*: in segmentation of the regenerating arms and the subsequent growth and patterning of the individual segments. Noteworthy, this expression pattern perfectly fits with the distalization-intercalary model described above, where the most distal tip and the proximal segments are those with a higher differentiation level (Czarkwiani et al., 2016). The *AfiBmp2/4* late expression is in contrast with the early expression observed in crinoids (see above), thus suggesting a large plasticity of the regulatory program of echinoderm regeneration.

Czarkwiani and co-workers (Czarkwiani et al., 2013, 2016) conducted studies on skeletal regeneration and identified dynamic patterns of expression of genes encoding transcription factors (*AfiAlx1*, *AfiEts1/2*, *AfiFoxB* and *AfiGataC*) and terminal differentiation genes (*AfiP19*, *AfiP58b*, *AfiClectin* and *Afiacoll*). Strong up-regulation of RNA levels of the transcription factor genes at early stage of regeneration, just before the skeletal primordia appears, has been shown by quantitative analysis. *In situ* hybridizations, revealed expression of these genes in discrete inner structures of the early regenerates, with exception of *AfiFoxB*, which was expressed in the epidermal layer. These data identify the presence of different cell types organized in various structures already in early regenerative stages of *A. filiformis*, further support the absence of a “true” blastema in this species. Later in advanced phases of regeneration the same transcription factor genes mark distinct mineralized tissues in proximal position: *AfiAlx1* is specifically expressed in spines and tube feet, *AfiEts1/2* is highly transcribed in cells that coincide with the shape and position of the vertebrae, and *AfiGataC* is primarily localized in the lateral shields. Interestingly all these genes are co-expressed in an area just under the distal tip of the late regenerates (50% regeneration) that corresponds to the highly proliferative zone that adds new segments to the regenerating arm (see Fig. 8; Czarkwiani et al., 2016). In contrast, the differentiation genes (*AfiP19*, *AfiP58b*, *AfiClectin*) are all identified in the dermal layer of regenerates in the advanced regenerative phase (stage 3-5 in Czarkwiani et al., 2016), and later in various skeletal elements.

An alternative strategy to characterize regulatory factors involved in regeneration was the use of classical PCR screens. Using the similar approach that was used in asteroids, eight fragments encoding *homeobox* transcription factors (five *Hox* and three *Hox*-related family) have been isolated from cDNA of regenerating tips of *A. filiformis* arms (Ben Khadra et al., 2014). They correspond to the anterior, medial and posterior groups of *Hox* genes, plus some *ParaHox* genes and a *Rough* orthologue. The presence of a complex set of regulatory genes of the body plan in the

regenerating arm of *A. filiformis* suggest their role in echinoderm regenerative processes, although more needs to be done to better characterize their expression domains and, thus, their precise roles.

A “blind” approach is has also been used in the context of ophiuroid regeneration, and this implies the characterization of transcriptomes. Recently, ophiuroids’ molecular studies have been extended to large gene expression data set with the aim to identify the whole complement of genes differentially regulated in different phases of the regenerative process and to understand the molecular basis of ophiuroid regeneration. An initial set of studies using a 9216 cDNA clones array showed that roughly 50% of the genes have a differential expression sometime during the *A. filiformis* regenerative process (Burns et al., 2011). Interestingly, in the early phase of regeneration a large set of genes associated with cell proliferation has been detected as well as the absence of TGF- β family genes, whereas in advanced phases a clear up-regulation of *BMP-1*, consistent with previous studies, has been reported. Furthermore, to address the molecular regulation of explant polarity, using a similar experimental set-up, genes differentially expressed in various body parts of 7 days post-amputation explants have been identified and compared with normal regenerating arms (Burns et al., 2012). Of particular interest are the signalling genes of the *Notch* and *Frizzled* related families, which are differentially upregulated in the distal part of the explant, suggesting involvement of *Wnts* and *Notch* signalling in establishing the intrinsic polarity of the explant. These genes may have a role also in regulating the regenerative proximal-distal axis. Furthermore, a very similar molecular make-up of the explant and normal regenerative part has been established, making the *A. filiformis* explants a very powerful tool for dissecting the molecular mechanisms underpinning echinoderm regeneration.

A combined proteomic and transcriptomic study on wound healing phases (1, 2 and 3 days post-amputation) of *A. filiformis* regeneration identified several hundred genes up- or down-regulated in injured arms, among them a sharp down-regulation of the signalling gene *TGF- β 2* and the muscle gene myosin heavy chain. At the protein expression level the biggest difference was seen in the regulation of the translation and integrin-mediated adhesion (Purushothaman et al., 2014). These data provide a molecular support to the observed remodelling of muscle cells and the cell migratory events described in the early phases of regeneration.

The molecular studies in *A. filiformis* provide a strong basis to understand the genetic control of Ophiuroidea regeneration; however, many questions remain open on the specific role of regulatory genes as well as signalling molecules involved in the explant polarity, in the initiation and progression of the regenerative process, and in the specification of various cell types. Finally, the

molecular studies should be coupled with cellular data to have a complete understanding of the origin of cells in the regenerate and the morphogenesis of the newly formed tissues to ensure a complete reestablishment of a functional arm.

5. Conclusions

Like in many other organisms, in echinoderms the ability to regenerate ultimately depends on the capacity to access a source of stem cells and/or to reprogram differentiated cells (Galliot and Ghila, 2010). Because the arm regenerative phenomena extensively involve the key contribution of progenitor cells present in the tissues, the obvious crucial and still unanswered issues pertaining to the regeneration research (Franco et al., 2013) are 1) stemness properties of responsible cells, in terms of origin and derivation (stem cells or reprogrammed cells), 2) activities (proliferation and/or migration), 3) plasticity and differentiation potential (final cellular phenotypes). All these cellular mechanisms are mediated by specific genetic pathway(s). However, very little is known about the origin/fate of cells contributing to the regenerated tissues, or the molecular regulators of the process, for almost any echinoderm, this being a paradox for a phylum well known for its striking regenerative abilities. Thus, the need of filling the gap of knowledge in our understanding of regeneration in stellate echinoderms, and, by extension, in all echinoderms is more and more evident. Such future projects will build on the thorough knowledge of the arrangement of cells in the regenerating arms but it will take a completely different set of approaches (*i.e.* electroporation, gene mapping, CRISPR-Cas9, cell tracking, knock-out/in experiments) to understand how these cells are specified during development and how their developmental programs are regulated by genes or gene batteries.

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