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The Sea Urchin Embryo: A Model for Studying Molecular Mechanisms Involved in Human Diseases and for Testing Bioactive Compounds

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Additional information is available at the end of the chapter

Abstract

Most of the current knowledge concerning fundamental genetic mechanisms, evolutionary processes and development, cellular physiology, and pathogenesis comes from studies of different animal model systems. Whereas mice, rats, and other small mammals are generally thought of as the typical model systems used by researchers in biomedical studies, aquatic models including both freshwater and marine organisms have long proved to be essential for the study of basic biological processes. For over a century, biologists have used the sea urchin embryo as a prototype for the investigation of developmental mechanisms that contribute to building the embryo body plan. Here we highlight the contribution of the sea urchin embryo as a simple model for studying aging and age-associated neurodegenerative diseases, as well as the pathways and mechanisms involved in cell survival and death. Moreover, we point out the role of this embryonic system as a potent and affordable tool for learning about developmental effects and toxicity responses to environmental contaminants and chemical compounds.

Keywords: embryonic model system, developmental effects, neurodegeneration, senescence, chemical compound assays

1. Introduction

Sea urchins are well-studied marine organisms belonging to the Echinoderm phylum. Since the mid-nineteenth century, the amazing transparent sea urchin embryo has been one of the favored animal models for descriptive experimental work on embryo development.

Afterward, it has been used for studying intercellular communication and cell adhesion [1–3], cell cycle control mechanisms [4], calcium signaling [5], fertilization [6], cell differentiation [7], and cell survival and death [8].

Added benefits of this system are low maintenance costs, small size, high fecundity, and the transparency of embryos, features that allow the direct observation of cell division and movement inside the living embryos and larvae. Moreover, as invertebrate species, sea urchins are not subject to restricted animal welfare concerns. Notably, this trait meets the strategy of the European Partnership for Alternative Approaches to Animal Testing for the development of alternative approaches to using animals in biological assays.

Studies on different sea urchin species have, indeed, identified maternal molecules which are spatially restricted and involved in the determination of cell fate [9–12], molecular mechanisms that respond to cellular stress such as heat shock [13–15], apoptosis [16], and autophagy [17]. More recently, researchers have used sea urchin embryos as a model for elucidating the role of cellular and molecular mechanisms involved in human health and disease. The potential of using the sea urchin as a model for disease research relies on the fact that general cellular properties are common to many organisms. The complete sequencing of the sea urchin genome has also revealed that sea urchins are more closely related to humans than other invertebrates [18, 19], including the model organisms *D. melanogaster* and *Caenorhabditis elegans* which are commonly used as disease models. Research on the sea urchin animal model now extends over a wide range of areas, such as immunology, microbiology, pathology, toxicology, and microbiology.

Recently the sea urchin embryo has played an important role as a model in the study of neurodegenerative disorders that can cause dementia and memory loss. This is possible because of the presence of a larval nervous system that arises during gastrulation within the ectoderm of the embryo [20]. Later, a set of neurons and neurites begins to be present in the structure called the ciliary band. Several clusters of neurons with associated neuropil are organized in ganglia, the largest of which is the apical organ of the larva, composed of some bilaterally positioned sensory cells containing serotonin. Thus, dysfunction in the cellular processes of these cells can be compared to what occurs in human neurons during pathological dysfunctions. In neurodegenerative disorders, such as Alzheimer's disease, neuronal loss occurs due to a buildup of a particular protein called Amyloid beta (Abeta) that, in this pathological condition, is misfolded and prone to aggregating, forming fibrils and plaques [21]. Abeta is a peptide derived from the proteolysis of a membrane-spanning precursor protein called amyloid precursor protein (APP); to strengthen the view that sea urchins can be utilized in these studies, an antigen related to human APP was identified [22, 23].

Another interesting opportunity presented by sea urchins is the possibility of gaining new insight into aging mechanisms. Aging is a multifactorial process, and many theories, such as telomere loss, oxidative stress, and free radical theories, have been proposed to explain this phenomenon at the molecular, cellular, systemic, and evolutionary levels [24]. Some reports indicate that different life spans have been found for different sea urchin species ranging between few years and 100 years. The sea urchin *Strongylocentrotus franciscanus*, indeed, shows a negligible senescence with decreased mortality and retained reproductive capacity

for a long time [25]. In contrast, *Lytechinus variegatus* has an estimated life span of only 4 years [26], while *Strongylocentrotus purpuratus* has an estimated maximum life span of more than 50 years [27]. Thus, to study sea urchin species with different life spans could be relevant for understanding mechanisms involved in aging.

Animal models also give an invaluable contribution to our knowledge of all biological aspects and processes, including the discovery and development of new chemical compounds by studying their properties and functions. The researcher's first choice is often to select models that are highly similar in biology and physiology to humans, although a combination of experimental data from different models can be more informative than those from a single organism, even evolutionarily closer. In vivo high-medium-throughput assays using small aquatic model organisms provide significant advantages for bioassay development, generating molecular and physiologically relevant responses. Among them, the sea urchin embryo is considered a valuable model to test and assess the safety of biomolecules, even those developed for therapeutic purposes [28–31] but also to discover basic molecular mechanisms and gene regulatory networks with which they may interfere.

In this chapter, we describe the role of the sea urchin embryo in studies of aging and age-related neurodegenerative disorders. Furthermore, we provide an update and underline the relevance of this model for assessing the developmental responses and/or toxicity effects of small molecules, many of which are used for medical purposes.

2. The sea urchin as a tool for studying neurodegenerative disorders

Neurodegenerative diseases are defined as hereditary and sporadic conditions, which are characterized by the progressive dysfunction of central or peripheral structures of the nervous system. These disorders are often debilitating disorders affecting memory, learning, skilled movements, and feelings. They include common diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) and rarer disorders such as multiple sclerosis, amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), spinocerebellar ataxia, and prion diseases. Even though their clinical manifestations and symptoms are completely different, neurodegenerative disorders share some common features, such as their appearance with aging, neuronal loss and synaptic anomalies, and mainly the presence of cerebral deposits of misfolded protein aggregates [32]. Although the presence of extracellular deposits is a common feature of these diseases, the protein component and distribution of the aggregates are specific to each pathology. Thus, for example, β-amyloid is the main component of the deposits in AD, α-synuclein in PD, and polyglutamine-rich in HD, and the accumulation of the protease-resistant aggregates of the prion protein (PrP) is present in the diverse forms of transmissible spongiform encephalopathy. The presence of abnormal aggregates in damaged regions of the brain is due to protein misfolding, which is one of the main causes of neurodegenerative diseases. Generally, the specific native protein is mainly composed of an unordered α-helical structure, but in the pathological condition, it is misfolded in a β-sheet structure that aggregates to form ordered protofibrils, fibrils, and plaques. Today, it is widely accepted that small oligomers, rather than compacted aggregates deposited in the brain, are

the real causes of neurodegeneration [33, 34]. However, regardless of the different sizes of aggregates, the induced toxicity leads to different cellular dysfunctions that culminate in the neuronal apoptotic process, though the mechanism by which protein misfolding and aggregation trigger neurodegeneration is still unclear. Since molecular mechanisms and basic pathways are conserved during evolution, the use of a simple model system can help us to understand the derived dysfunction and the pathways affected by a specific insult. Among the neurodegenerative diseases, AD is a pathology that is rapidly increasing with longer human life expectancy. AD is, indeed, the most common cause of dementia in elderly people and typically begins with subtle and weakly recognized failures of memory and slowly becomes more severe. It leads to the progressive loss of mental, behavioral, and functional abilities. The pathological hallmarks in the AD brain present two different abnormal structures, extracellular amyloid plaques, composed of amyloid-β (Aβ) peptides, and intraneuronal neurofibrillary tangles, composed of hyperphosphorylated tau [21]. Amyloid-β peptides (Aβ) are proteolytic fragments of 40–42 amino acids, derived from the transmembrane amyloid precursor protein (APP), whereas tau is a brain-specific, axon-enriched microtubule-associated protein. Depending on cellular conditions, Aβ is misfolded, and the establishment of ordered structures rich in β-sheet, prone to self-assembling, produces aggregates typical of amyloid assemblies. For a long time, large aggregates of Aβ, found in amyloid plaques, have been considered the major cause of neuron damage and degeneration in AD. Recent studies have, instead, demonstrated that Aβ soluble oligomers, also known as Abeta-derived diffusible ligands (ADDL), are the more toxic form [35]. The combination of biochemistry, molecular and cell biology, and systems biology has been utilized for understanding some of the main molecular mechanisms leading to AD onset, but other efforts and knowledge are required. AD is probably caused by complex interactions among multiple genetic, epigenetic, and environmental factors; thus, interdisciplinary approaches leading to the identification of useful biomarkers are necessary for the development of suitable drugs for prevention and treatment.

In this scenario, how can a simple model system help AD neurodegeneration research? What questions can we ask and what answers can we have from a simple model system? For this aim, the first issue to be addressed is to obtain evidence that Aβ induces toxicity on the chosen model system. As mentioned earlier, the sea urchin is a suitable model system to test effects induced by chemical or natural toxic agents on biochemical pathways and embryo morphology. The sea urchin has a primordial nervous system, and the same neurotransmitters used in embryonic development are conserved between sea urchins and the mammalian brain. The sea urchin embryo, indeed, synthesizes, stores, and releases acetylcholine (Ach) and other neurotransmitters and possesses analogous receptors and downstream signaling cascades, all of which appear over a well-defined developmental period and act as morphogens in controlling cell differentiation and embryo and larva assembly [36]. Furthermore, under specific stimuli, the sea urchin embryo triggers apoptosis [16], the death mechanism by which neurodegeneration occurs (**Figure 1**). Studies from different laboratories have utilized two sea urchin species, *Paracentrotus lividus* and *Sphaerechinus granularis*, to test Aβ toxicity.

By using the sea urchin as model system, one of the first questions to be addressed was to establish if different structural Aβ species have the same toxicity. To this aim, it was produced a recombinant $A\beta_{42}$ peptide by using the pQ30 expression vector [37]. Different sizes
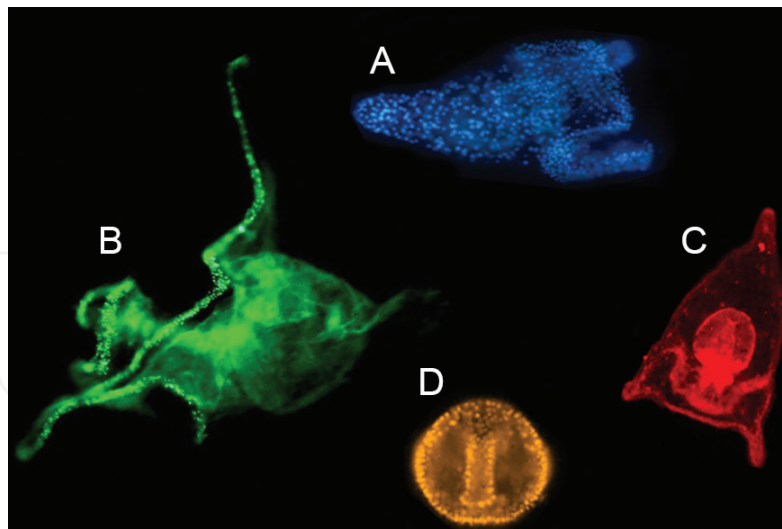
Figure 1. Sea urchin embryos have neurons and neurites, and respond to classical mechanisms involved in neurodegeneration processes, such as apoptosis or oxidative stress. *P. lividus* gastrula and pluteus stage embryos are depicted. (A, D), nuclei of pluteus and gastrula stage embryos are stained with Hoechst 33258; (B), late-stage larva stained with anti U-295 antibody (kind gift of Dr. David R. McClay) that specifically recognizes neurites at the ciliary band; (C) reactive oxygen species detected by a fluorescent dye in a pluteus stage embryo.

of aggregates, either small oligomers or larger aggregates, were obtained upon dissolving the recombinant $A\beta_{42}$ under different pH conditions (7.2 or 3, respectively); different ionic strengths, indeed, change the kinetic of oligomer formation. By using a light scattering instrument, it was established that at pH 7.2, small $A\beta_{42}$ species were present in solution; in contrast, at pH 3, larger aggregates were detected. To define a structure-toxicity relationship, $A\beta_{42}$ was dissolved in the two solutions and administered, in different concentrations, to a two-cell-stage embryo culture of *Paracentrotus lividus.* Under these conditions, embryos were allowed to develop until controls had reached the pluteus stage, and different percentages of surviving or degenerating embryos, with respect to the controls, were observed. After only 4 h of embryo development, morphological defects in the cell membrane were observed. After 1 day of development, instead, retardation of embryo development as well as cellular disorders visible inside the blastocoele was detectable. After 48 h of development, cellular degeneration in two different pathological phenotypes, the occluded blastulae and the occluded prism, was present [37]. In general, small oligomers were more toxic than larger aggregates, in agreement with the discovery that small ADDLs are highly toxic [35], indicating that the $A\beta_{42}$ state of assembly appeared to influence its biological activity. Based on the obtained results, it was supposed that more diffusible small oligomers could be more easily internalized than larger aggregates in the developing embryo, producing cellular and molecular dysfunction. Thus, the sea urchin was a suitable in vivo organism for $A\beta_{42}$ toxicity studies and could be used as an indicative tool for the pharmacological evaluation of a therapeutic agent.

Regarding $A\beta_{42}$ toxicity, another group identified both the critical periods in which different types of anomalies are induced by $A\beta_{42}$, and the protective role played by acetylcholine and other neurotransmitters such as serotonin and cannabinoids [38]. The sensitivity to $A\beta_{42}$ is

greatest when the peptide is introduced at the mid-blastula stage, in a particular period called "mid-blastula transition," when new genes become expressed by the embryonic genome. Thus, the neurotoxic effects of Aβ are associated with changes in gene transcription. Further, the morphological changes correspond to a level of toxicity indicating that sea urchins provide a system that allows for the rapid screening of potential therapeutic interventions [38]. Furthermore, the morphological anomalies were inhibited by the addition of lipid-permeable analogs of acetylcholine (arachidonoyl dimethylaminoethanol), serotonin (arachidonoyl serotonin), and cannabinoids (arachidonoyl vanillylamine), indicating that they can prevent the neurotoxicity associated with Aβ and be used for therapies that enhance cholinergic function, as well as for AD.

By using *S. granularis*, the effect of a specific 15-amino acid sequence (NWCKRGRKQCKTHPH) placed in positions 96–100 within the extracellular domain of the APP protein (APP$_{96–110}$) was evaluated and compared to that of Aβ$_{42}$ [39]. This fragment corresponds to a proteoglycan-binding domain that specifically controls neurite outgrowth and other aspects of neurodevelopment [40]. Sea urchin embryos at the 2–4 cell stage, blastula stage, or at late gastrula to early pluteus stages were treated with the two peptides, with or without neurotransmitters or neurotransmitter-related agents, and embryonic malformations were observed. With respect to Aβ$_{42}$, APP$_{96–110}$ had a weaker effect in disrupting development, requiring higher concentrations to produce the same malformations caused by Aβ$_{42}$. APP$_{96–110}$ was dangerous only within a defined window of vulnerability corresponding about to the mid-blastula stage, whereas Aβ$_{42}$ had adverse effects from early cleavage to the pluteus stage [39]. For both peptides, developmental anomalies were prevented or reduced by the addition of neurotransmitters. This finding indicated a role for APP in development and identified specific interactions with neurotransmitter systems that act as morphogens in developing sea urchins as well as in the mammalian brain.

3. The APP gene is conserved in the sea urchin genome

As mentioned above, Aβ is a peptide derived from a multiple proteolytic cleavage at the C-terminal position of a large transmembrane protein called APP [21]. To validate the sea urchin embryo as a model system in the study of Aβ-induced toxicity, it was necessary to demonstrate the presence of an antigen related to human APP. APP is a protein conserved during evolution, and APP-related genes have also been found in less evolved organisms, such as *Drosophila melanogaster* and the worm *Caenorhabditis elegans*. This suggested that common mechanisms and responses to pathological stimuli could be conserved [41–43]. Thus, the possible presence of an antigen related to APP in sea urchins was investigated. For this aim, the presence of APP was examined in proteins extracted from gastrula stage embryos and pluteus larvae at 48, 120, and 288 h of development [22]. The choice of these developmental stages was based on the moment at which a primordial nervous system appears. The sea urchin larval nervous system consists of an array of neurons that control swimming and feeding, and a more defined organization begins to be present at the late gastrula stage [20].

The presence of an antigen related to APP, called *Paracentrotus lividus* APP (*Pl*APP), was detected, and in addition, a fragment of lower molecular weight was identified at late pluteus stage, suggesting that after the gastrula stage, a portion of the *Pl*APP was proteolytically cleaved, producing a peptide as occurs in higher organisms. Immunofluorescence colocalization experiments with serotonin, a neuronal antigen, confirmed that *Pl*APP was present in sea urchin primordial neurons [22]. Furthermore, clear proof of the presence of a gene encoding for *Pl*APP was obtained by cloning and sequencing a full-length cDNA.

Subsequently, the possibility that *P. lividus* embryos could trigger different apoptotic pathways was investigated after stimulation with Aβ aggregates of different sizes. Aβ-induced apoptosis generally occurs through caspase-dependent pathways, even if caspase-independent pathways have also been described [44, 45]. In sea urchin embryos, apoptosis is never found during cleavage stages. It begins to manifest between the early blastula and late gastrula stages [16, 46]; thus, we performed apoptosis analysis only in embryos after the gastrula stage. In light of this evidence, *Paracentrotus lividus* embryos at the two-cell stage were incubated with small oligomers or fibrillar aggregates and cultured until the controls arrived at gastrula and pluteus stages [22]. By microscopic inspection, a higher percentage of malformed or dead embryos was observed upon treatment with Aβ oligomer forms with respect to the Aβ aggregate forms. By TUNEL assay, apoptosis on surviving embryos treated with the different aggregation forms was detected. In addition, it was found that aggregates employed an exclusively extrinsic apoptotic pathway via caspase 9 activation, whereas oligomers activated both extrinsic and intrinsic apoptotic pathways via caspase 9 and caspase 8, respectively. These findings suggested that a part of the smaller soluble oligomers was able to penetrate the cells and produce mitochondrial damage that activated caspase 9. In contrast, other oligomers could attach to a neuron surface-specific binding site and, perhaps, seed the aggregation process, mimicking what occurs in human pathology. In summary, these studies of the sea urchin embryo give information about the possibility that small oligomers may cross the membrane and penetrate the intracellular environment. This appears to be in agreement with biophysical and modeling studies that have demonstrated the ability of Aβ to interact with a lipid bilayer due to its obliquity and hydrophobicity [47].

4. Species-specific sea urchin longevity and senescence

Aging, also called senescence, is the process of becoming older. The term is particularly used to refer to humans, but all living organisms generally share this process. It is a well-defined biological route characterized by a progressive functional decline and increasing death over time. Because aging and life span characteristics diverge extremely between similar species, it has been believed that intrinsic organism life span is genetically determined and developed through an evolutionary process similar to the one that determines other species-specific characteristics. Furthermore, many theories have been proposed to explain this phenomenon at different levels such as loss of telomeres or enhanced free radical generation [24]. Telomeres are chromosome regions made up of repetitive sequences which function to protect chromosome deterioration. During aging, telomeres become shorter, and their attrition can be

counteracted by telomerase activity. Thus, telomere attrition contributes to aging. The free radical, or oxidative stress, theory of aging, instead, proposes that the accumulation of oxidative cellular damage is a major contributor to the aging process and a key determinant of species longevity. The latter, referred to especially long-lived members of a population, is the challenger of aging. Studies on human longevity suggest that some of it is attributable to genetic factors and the rest is influenced by epigenetic and environmental factors [48]. Lifestyle and nutrition influence longevity at all stages of development and levels of human diversity [49, 50].

However, there are a number of different animals that show slower senescence, with decreased mortality and no reduction in reproductive capacity, no increase physiological dysfunction, and increased disease resistance with age [51, 52]. The study of these animals may furnish new insights about effective defenses against the degenerative process of aging. The sea urchin offers an ideal model to investigate mechanisms of longevity and reduced senescence. Different species of sea urchins, indeed, have very different natural life spans, ranging from 4 to more than 100 years, thus providing a unique model to investigate the molecular, cellular, and physiological mechanisms underlying both life span determination and negligible senescence. As told before, the species *Strongylocentrotus franciscanus* lives in excess of 100 years [25, 53]; *L. variegatus* has a life span of only 4 years [26], while *S. purpuratus* has an estimated life span of more than 50 years [27]. Identification of molecules involved in specific pathways that could be activated or inhibited in species with different longevity may provide insight into mechanisms involved in senescence. Thus, sea urchins represent an interesting alternative model for aging research [52]. To demonstrate that the red sea urchin, *S. franciscanus*, is a long-lived organism, some studies were carried out in two different localities of Washington State, USA [53]. By using a chemical marker, tetracycline-HCl, that binds to calcium ions and becomes incorporated into the skeleton during calcification [54], growth rates were determined. Further, since aging can be defined as the time-related deterioration of the physiological functions necessary for survival and fertility, these parameters were analyzed. Some individuals were tagged, and diameters (diameter test) of all sea urchins and their gonads, as survival and fertility parameters, were measured at the time of tagging as well as when collected after a year or more in the field, to establish the size structure of the populations. Gonad size, indeed, changes during the seasons, reaching a maximum in spring and minimum in summer, and these different sizes are correlated to reproductive capacity.

No decrease in gonad size with respect to the increasing diameter of individuals was found. By applying a mathematical model, change in survival and increasing reproductive esteems were done, and it was concluded that *S. franciscanus* shows no evidence of senescence. This was in contrast with the disposable soma theory [55], in which generally organisms have a limited amount of metabolic resources that have to be used to maintain the reproductive and nonreproductive activities of the organism (soma), including the repair of cellular damage. Senescence occurs when metabolic resources are exhausted and survival mechanisms, which operate throughout life, are altered. The data obtained on *S. franciscanus,* the long-lived species, indicate that it is subject to negligible or slow senescence [51] or none at all. As related above, life span differences among different species of the same organism have been found. This means that the biological changes leading to senescence occur at different ages, and

comparison among long-lived (*S. fraciscanus*), intermediate-lived (*S. purpuratus*), and short-lived (*L. variegatus*) species could give new insights regarding the molecules, mechanisms, and key cellular pathways involved in life span determination and aging.

With the aim of finding the biomarkers involved in the longevity/senescence process, a proteomic study has been carried out using coelomic fluid of the three sea urchin species [56], whose age was determined by diameter test. Similarly to the blood, the coelomic fluid of sea urchins contains a miscellany of cells and macromolecules that provide essential functions such as nutrient transport as well as immune and clotting activities. Further, it contains proteins that are actively secreted as well as proteins released through cell lysis and cellular turnover. Proteomic analysis of the three sea urchin species revealed that the ectodomain of low-density lipoprotein receptor-related protein 4 (LRP4) was among the proteins that mainly increased with age. It has been proposed that since LRP4 is considered to be involved in Wnt signaling, the role of Wnt in negligible senescence should be better investigated [56].

5. Senescence and oxidative stress

According to the oxidative stress theory, both of the processes of aging and longevity are regulated by the accumulation of reactive oxygen species (ROS). ROS are partially reduced derivatives of oxygen that are highly reactive with major cell components such as proteins, lipids, and DNA, causing their damage. Many physiological cellular processes generate ROS, but others are produced by exposure to various external stimuli, such as ultraviolet light, ionizing radiation, and environmental toxins. Oxidative stress results from an imbalance between the production of ROS and the cell's intrinsic ability to inhibit damage through the production of antioxidant molecules or mechanisms that repair or eliminate damaged molecules. Thus, it is an imbalance between oxidants and antioxidants in favor of oxidants, which probably leads to damage. The existence of sea urchin species with different natural life spans, including some species with extraordinary longevity and negligible senescence, represents a model to study the accumulation of cellular oxidative stress with age. Oxidative cellular damage, antioxidant capacity, and proteasome enzyme activities were measured in different tissues of the three sea urchin species with different life spans, *L. variegatus, S. purpuratus*, and *S. franciscanus* [57]. No aged-related change in the marker of oxidative damage was observed in tissues of sea urchins with different life spans. Furthermore, levels of 8-hydroxy-2-deoxyguanosine, a marker of oxidative DNA damage, measured in cell-free coelomic fluid showed no general increase with age. Thus, the results suggested that negligible senescence is accompanied by a lack of accumulation of cellular oxidative damage with age; thus, the maintenance of antioxidant capacity and proteasome enzyme activities may be an important mechanism to mitigate time-linked damage.

Oxidative stress and mitochondrial dysfunction are the basis of aging and, consequently, to neurodegenerative diseases including AD. Thus, the use of natural antioxidants suppressing or reducing oxidative stress could be a neuroprotective strategy for blocking cell death. Ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid) is an antioxidant naturally present in plant cell walls. It has a phenolic nucleus and a long side chain, so it readily forms a resonance-stabilized

phenoxy radical with high antioxidant [58] and anti-inflammatory [59] activities. It has been suggested that FA can act as a free radical scavenger [60]. Ferulic acid was used in vitro to block damage induced by beta amyloid, in the presence or not of drug delivery systems that could improve its transport and release in an in vivo system [61, 62]. *P. lividus* embryos were used as models to test the ability of FA to reduce cytotoxicity induced by Abeta. By morphological and fluorimetric test, it was possible to demonstrate that FA reduced the number of perturbated embryos induced by $A\beta_{42}$ [63]. Under this stimulus, sea urchins induced ROS generation and mitochondrial dysfunction, leading to embryo degeneration, and this process was counteracted by FA addition. Furthermore, after $A\beta_{42}$ treatment, a modulation of the molecules involved in the apoptosis process and activated by oxidative stress, such as the transcription factor foxo3a, was observed. In agreement with its antioxidant role, FA was able to inhibit the degenerative process through the downregulation of foxo3a. As happens in higher organisms, the sea urchin embryo exploits molecules, pathways, and mechanisms involved in both survival and death processes.

6. Sea urchin cells and embryos respond to external agents: humans exploit their skills!

Echinoderms, together with the Hemichordates, are sister groups of the Chordate phylum which includes humans and other vertebrates. As revealed by the analysis of the genome of the sea urchin *S. purpuratus*, the first nonchordate marine deuterostome to be completely sequenced [18, 64–66], a significant amount of the sea urchin gene repertoire is genetic material exclusive to the deuterostome superclade. Notably, a large part of the human gene catalogue, including orthologs of many human disease-associated genes, is expressed in sea urchins. Genome drafts, together with transcriptomes and other expression data from sea urchins and other Echinoderm species, are available at the SpBase and Octopus websites (http://www.echinobase.orghttp://www.echinobase.org/Echinobase/ and http://octopus.obs-vlfr.fr/, respectively).

A large number of studies have highlighted the power of the sea urchin embryonic system. The discovery of conserved gene regulatory networks (GRNs) driving development and morphogenesis has been a milestone in biology. Interactions between sequence-specific DNA binding molecules and DNA regulatory regions, together with signaling interactions and cofactors, control transcription and gene spatial temporal and expression [67–71]. Development, cell-type specification, and differentiation depend on these hardwired processes. A canonical and widespread developmental event, the embryonic epithelial mesenchymal transition (EMT), has been intensely studied in sea urchins. Primary mesenchyme cell (PMC) ingression during embryogenesis is evolutionarily conserved and is an excellent model of EMT in vivo [72, 73]. In humans, reactivated EMT drives organ fibrosis and tumor progression [74–76]. The approach to the identification of the GRN and subcircuits controlling EMT in sea urchin embryos constitutes a beautiful example of the strength of this system, as an excellent model system for the analysis of the transcription factors controlling EMT [77]. In addition, more traditional approaches have helped to discover networks and signaling pathways and even highlight the contribution of single molecules participating in embryo patterning along the embryonic axes [78–86].

The US National Institutes of Health has designated the sea urchin embryo as a model system helping to explain processes related to human health and disease [18]. Medical research and studies on environmental contaminants rely on the development of simple and affordable bioassays in cell systems and model organisms. These experimental activities are essential to elucidate mechanisms through which chemical compounds may exert their effects. Sea urchin embryo assays show high sensitivity and experimental reproducibility, providing rapid evidence of gamete abnormalities, developmental defects, and molecular changes in gene transcription and signaling pathways involved in constructing the embryo. The activity of several chemical compounds has been evaluated using sea urchin embryos along their early developmental stages, allowing the use of hundreds or even thousands of embryos in single screens. The high-throughput properties of this system benefit from the large amount of molecular and evolutionary data in the literature joined to the availability of powerful molecular tools that researchers of the sea urchin scientific community exchange each other [87].

Sea urchins express gene families that participate in response to environmental stressors. A genome-wide survey of the chemical defense gene network in *S. purpuratus* revealed around 400 genes, whose products contribute to the response to environmental stressors and cellular homeostasis. They include genes encoding for cytochrome oxidases, conjugating enzymes, ATP-dependent efflux transporters, oxidative detoxification proteins, and transcription factors involved in their regulation, many of which are expressed during embryogenesis. A great part of this repertoire is extraordinarily conserved during evolution [88, 89].

Members of the ATP-binding cassette (ABC) superfamily transport-specific molecules and substrates across membranes and mutations in these genes contribute to several human genetic disorders [90]. In sea urchin embryos, efflux transporter genes act as a first line of defense against toxic xenobiotic compounds; their expression is upregulated after fertilization [91], and this process is accomplished with relatively small energy costs [92, 93]. Cytochrome P450 family (CYPs) enzymes catalyze the oxidation of many xenobiotics, environmental chemicals, and drugs and protect the embryo from toxicants. Cytochrome P450-dependent oxidase activities have been used as potential markers for the assessment of environmental quality in marine areas, using different Mediterranean species including the sea urchin *P. lividus* [94].

In the sea urchin embryo, inducible forms of the heat shock proteins HSP70 (HSP70/HSP72) mainly mediate the cellular stress response. In sea urchin embryos, HSPs were first identified after heat treatment [14], although their expression has also been induced by heavy metals [95], UVB radiation, and other environmental pressures [96, 97]. It has been shown that proteins involved in stress responses cross talk with the immune signaling pathways [98]. In contrast to mammals that use both adaptive and innate immune responses, sea urchins only developed innate immune functions, as testified by the presence of an immune system with a rich repertoire of recognition receptors, regulators, and effectors [99]. This complex repertoire provides a nonspecific response to infection and/or injury [100, 101]. Immune receptor genes include molecules participating in the recognition of pathogens. Toll-like receptors (TLRs) and the echinoid-specific 185/333 gene family are expressed in different classes of adult immune cells, known as coelomocytes, while NACHT domain-LRR (NLR) cytoplasmic receptors have been found in the gut epithelium. Another expanded gene family present in the sea urchin genome encodes for the scavenger receptor cysteine-rich (SRCR) proteins [89, 102–104]. The

expression of many of these factors is also activated during the larval stages and is mediated by a heterogeneous set of specialized immune cells [105, 106]. The adaptive behavior of the powerful sea urchin immune cells and the modifications of their parameters have been recently used as tool for monitoring disease susceptibility and to establish water quality standards and for nano-safety/nano-toxicity analyses [107–109]. In comprehensive review articles about the use of sea urchin embryos for assessing the toxicity of environmental contaminants, the chemical and physical effects of stress during development or in adults are reported in a thematic book focused on the molecular marine biotechnology of Echinoderms [110].

Methods for assessing pharmaceutical and pollutant embryo toxicity and teratogenicity in sea urchin embryos have been developed in sea urchins, indicating that gametes and embryos function as sensitive indicators of environmental toxicity and mutagenicity. Several biological endpoints can be simultaneously evaluated [111, 112], and the long history of sea urchin developmental biology has enormously contributed to these studies.

Teratogenic effects of lithium on sea urchin embryos were first reported more than one century ago [113]. After treatment with LiCl, sea urchin embryos developed as exogastrulae, characterized by an increase in endoderm and mesoderm tissues at the expense of ectoderm. The most visible sign was the alteration in the balance between animal and vegetal regions, with an abnormal and exaggerated presence of vegetally derived cells [114, 115]. It was later shown that embryos cultured in high Li+ concentration delayed the cell cycle, arresting cells at metaphase and at cytokinesis, and that lithium affected the phosphoinositide cycle [116, 117]; this block was, in fact, reversed either after removing lithium or by counteracting its effects by adding myoinositol [118, 119]. Molecular analyses have added information on the properties of lithium, which shift the border between the vegetal and animal embryonic regions. Lithium dramatically increased the expression of vegetal-specific molecular markers [120], even in isolated animal caps [121], and restricted the expression domain of the hatching enzyme toward the animal pole [122, 123]. Lithium, which has been successfully employed in the treatment of bipolar disorders [124], mimicked the activation of Wnt/beta-catenin signaling, producing embryonic phenotypes similar to those elicited by GSK3β inhibition [80]. More recently, it has been reported that sea urchin female gametes had enhanced sensitivity and embryo development was more affected when LiCl was applied before rather than after fertilization [30].

The metallic element nickel perturbs the sea urchin embryo dorsoventral axis, altering the commitment of ectodermal cells. Embryos treated with $NiCl_2$ showed an overrepresentation of oral (ventral) ectoderm cells, causing an increase in the expression of the oral ectoderm-specific markers EctoV and *Orthopedia* [125, 126].

The effects of specific inhibition of the epidermal growth factor receptor (EGFr) are another example of the strength of sea urchin embryonic assays to analyze perturbations at cellular and molecular levels. EGFr inhibition by Tyrphostin AG1478 determined the decrease of mesoderm and endoderm marker expression, the ectopic distribution of the ectoderm-specific hatching enzyme, and the reduced level of β-catenin nuclearization; effects on development were mediated by the MAPK-ERK signaling pathway. The addition of TGF-alpha ligand of EGFr to AG1478-treated embryos completely rescued the embryonic phenotypes either at

early or late developmental stages, indicating that the effects of this compound were EGFr-specific (**Figure 2**). Furthermore, AG1478 negatively controlled the expression of the EMT-linked transcription factors Snail and ScratchX/Snai2 and inhibited primary mesenchyme cell specification and EMT [31, 127].

The sea urchin embryo has also been used as model for the assessment of antiproliferative, antimitotic, and cytotoxic effects of small molecules. Due to the structural and functional similarities of tubulins among species, including sea urchins [128–130], a number of chemical compounds
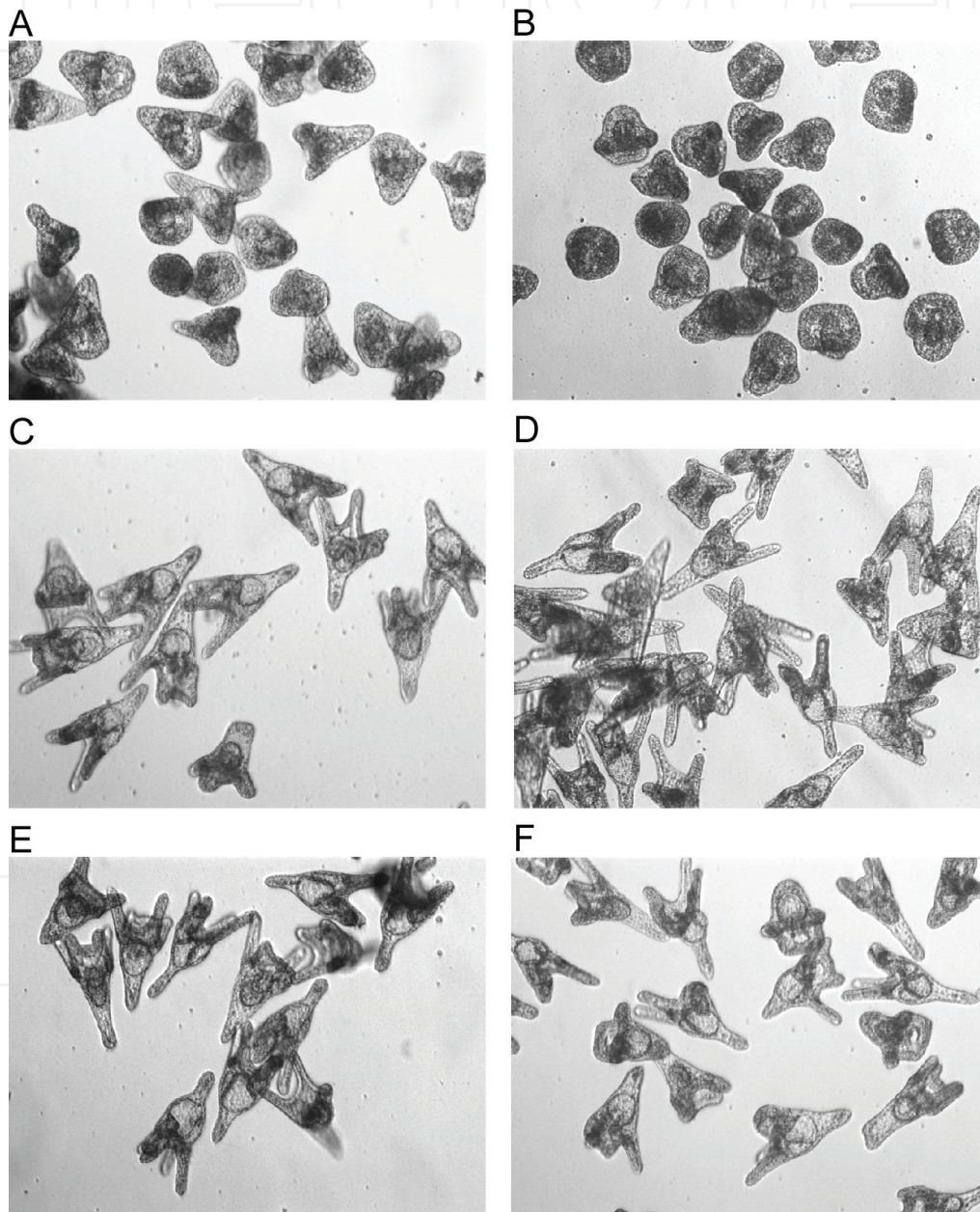
Figure 2. Effects of EGFr inhibition and developmental rescue by TGFα. (A,B) 48 h pluteus stage embryos treated with 10 μg/ml AG1478 from fertilization or from hatching blastula stage, respectively. (C, D) Embryos co-treated with 10 μg/ml AG1478 and 80 ng/ml TGFα. (E) Plutei after treatment with 80 ng/ml TGFα; (F) control plutei.

have been screened for their antimitotic and microtubule-destabilizing activity. The specific effects on embryo development and the swimming behavior of *P. lividus* embryos allowed the identification of chemical agents with specific tubulin destabilizing effects [131–134].

The sea urchin embryo, from fertilization to late embryonic stages, was the subject of several assays aiming to test antitumoral and antiepileptic compounds such as doxorubicin, retinoic acid, phenytoin, valproate, and tamoxifen. Straightforward, reproducible morphological analyses demonstrated that sea urchin embryos function as a simple, specific, and sensitive biological factory for developmental toxicology experiments [135–138]. Reports describing the toxic effects of chemicals, pharmaceuticals, insecticides, pesticides, and other environmental contaminants on sea urchin embryos and adults have been recently published [112, 139–143]. Investigators in marine research have paid particular attention to the fast growth of submicroscopic materials contaminating marine and coastal ecosystems and have analyzed biochemical and histochemical markers of toxicity, taking advantage of the sensitivity of sea urchin gametes, embryos, and adult cells [144]. Recently, researchers have developed new computation methods and a new toxicity index, integrating the frequency of abnormal embryos obtained after treatment with marine waters and sediments with the adverse effects observed during development. This approach has been taking advantage of the high number of morphological parameters that can be measured in sea urchins [145].

Finally, sea urchins play an essential role in ecology, being part of marine benthic communities and functioning as grazers and prey [146]. The physiological performance of marine organisms in challenging the natural environment is endangered by old and emerging hazards coming from anthropic activities and climate-induced changes [147]. Worldwide, living sea urchins, as part of trophic cascades and an important fishery resource, may become threatened in the near future. Assessing the adaptive capacity of these organisms, also in the context of multi-stressor effects, to function as early warning sentinels is thus of increasing interest to researchers and marine organizations, for the management of biological resources and ecosystems [148].

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