Archives of Biochemistry and Biophysics 474 (2008) 167–174

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

EGFR signalling is required for *Paracentrotus lividus* endomesoderm specification

Daniele P. Romancino [a], Giovanna Montana [a], Vincenzo Cavalieri [b], Giovanni Spinelli [b], Marta Di Carlo [a,*]

[a] *Istituto di Biomedicina ed Immunologia Molecolare (IBIM) "Alberto Monroy", CNR, sez. Biologia dello Sviluppo, via Ugo La Malfa 153, 90146 Palermo, PA, Italy*
[b] *Dipartimento di Biologia Cellulare e dello Sviluppo (Alberto Monroy), Università di Palermo, Parco d'Orleans II, 90128 Palermo, Italy*

ARTICLE INFO

Article history:
Received 5 February 2008
and in revised form 6 March 2008
Available online 26 March 2008

Keywords:
EGFR
A/V axis
Endomesoderm
Skeletogenesis
Sea urchin
AG1478

ABSTRACT

The EGFR pathway is critical for cell fate specification throughout the development of several organisms. Here we identified in sea urchin an EGFR-related antigen maternally expressed and showing a dynamic pattern of localization during development. To investigate the role played by the EGFR in *Paracentrotus lividus* development we blocked its activity by using the EGFR kinase inhibitor AG1478. This treatment produces decrease of EGFR phosphorylation, and embryos with various defects especially in the endomesoderm territory until to obtain an animalized phenotype. These effects are rescued by the addition of TGF-α, an EGFR ligand. The role played by EGFR-like along the animal/vegetal axis was also detected, after AG1478 treatment, by the extended distribution of HE and decreased nuclearization of β-catenin in vegetal cells. Moreover, inhibition of EGFR-like reduced ERK phosphorylation, necessary for cell fate specification in the micromeres and their derivates. Taken together these results indicate that EGFR-like activity is required both for A/V axis formation and endomesoderm differentiation.

Intracellular signalling plays a significant role in the regulation of biological processes such as cell proliferation, differentiation, migration and survival. Signals can either stimulate or inhibit a specific process, and the same signal can have different effects during different development events, even within the same organism. Moreover, in order to generate specific responses, distinct signals can cross-talk between one another, and tissue-specific downstream signalling components can also be employed.

The epidermal growth factor receptor (EGFR)[1] is a transmembrane receptor tyrosine kinase (RTK) that acts through the Ras/MAP, or PLCγ–PKC, or IP3R or cyclin-dependent cascades and mediates various inductive signalling events in several tissues to regulate normal embryonic development and adult differentiation [1–4]. It is an important element in other signalling pathways, and its role is not exclusively limited to that of the EGF reception, but is considered a mediator of different signalling systems and as a cellular communication network switching point [5]. EGFR is, indeed, subjected to regulation and modulation by multiple ligands. The known ligands for EGFR include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), epiregulin (EREG), β-cellulin (BTC), and heparin-binding EGF (HB-EGF) [6]. Such ligands share a common EGF domain, permitting them to form a specific secondary structure motif, while they share no or very little homology outside this domain. Sequences inside and outside the EGF domain determine the specificity of any given ligand. Signalling via EGFR is important during the development of mice, fruit flies and nematodes, and its mutation or enhancement leads to human tumor progression.

A family of EGF receptors exists in mammals, including ErbB1, ErbB2, ErbB3 and ErbB4. Ligand binding to ErB receptors induces the formation of receptor homo- and heterodimers and activation of specific signalling pathways [2,7]. Overexpression of this receptor and its family members has been correlated with development of different human tumors [8].

EGFR in *Drosophila* is present at many different stages of development and its signalling activity is modulated by activating or inhibiting its four ligands gurken, spitz, vein, argos [9–14]. Further, it has been demonstrated that EGFR controls invagination of epithelia in a process called "boundary smoothing", that permits to convert flat epithelium into three-dimensional organs [15].

In *Caenorhabditis elegans*, the EGFR family member LET-23 is present in several tissues and is required in vulva differentiation, acting on tissue-specific transcription factor such as LIN-31 [16–18]. A recent discovery has demonstrated that activation of EGFR

* Corresponding author. Fax: +39 0916809548.
E-mail address: di-carlo@ibim.cnr.it (M. Di Carlo).

[1] *Abbreviations used:* AP, alkaline phosphatase; ASW, artificial sea water; BCIP, 5-bromo-4-chloro-3-indoyl phosphate; DAB, diaminobenzidine; DIC, differential interference contrast; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EGIP, exogastrulation-inducing peptide; ERK, extracellular signal-regulated kinase; ETM, epithelial mesenchymal transition; FGF, fibroblast growth factor; GRN, gene regulatory network; HE, hatching enzyme; MAP; mitogen-activated protein; NBT, nitroblue tetrazolium; PABA, p-aminobenzoic acid; PDGFR, platelet-derived growth factor receptor; PI3K, phosphatidylinositol 3-kinase; PKC, phospholipase kinase C; PLC, phospholipase C; PMC, primary mesenchyme cells; PMSF, phenylmethanesulphonylfluoride; RTK, receptor tyrosine kinase; SMC, secondary mesenchyme cells; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; TGF-α, transforming growth factor-α; VSM, vegetal signalling mechanism.

0003-9861/$ - see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.abb.2008.03.022

within a single neuron, ALA, is sufficient to induce a quiescent state. This pathway modulates the termination of locomotion that normally occurs during the lethargic period that precedes larval molting. Thus, EGF signalling plays a role in the regulation of behavioral quiescence [19].

The sea urchin embryo is a regulative developmental system whose patterning is established though a combination of maternal determinants and signalling among cells and/or tissues [20]. Ectoderm, mesoderm and endoderm fates along the A/V axis are determined by an inductive process, starting from the vegetal half of the embryo and initiated by cell-autonomous activation of canonical Wnt signalling in micromeres. Thus, specific signals constitute an input to transcribe specific spatial regulatory genes that are organized in network [20,21].

The sea urchin represents a good model system for studying changes in developmental programming due to the perturbation of specific signalling pathways. Two epidermal growth factor (EGF)-relate genes from *Strongilocentrotus purpuratus,* which encode putative secreted proteins have been cloned and characterized, suggesting the presence of a receptor [22–24]. Moreover, the presence of EGFR in the sea urchin species *Lytechinus variegatus* has been suggested by the recognition of a specific protein by antibodies against the mammalian receptor, and its involvement in gut and spicule formation has been demonstrated [25].

In an effort to identify the signalling pathways involved in early *Paracentrotus lividus* development, particularly, the moment in which cells begin to acquire distinct tissue-specific properties, we decided to investigate the possible involvement of an EGFR-like in this role by inhibiting its function with AG1478 [26]. Here we show that EGFR-like activity is required both for A/V axis formation and supporting the roles played by the micromere–PMC lineage in order to achieve correct endomesoderm development.

Materials and methods

Morphogenetic assay

Eggs from sea urchins (*P. lividus*) were demembranated by fertilization in 2 mM PABA. Approximately 1000 embryos were added to artificial sea water (ASW) containing Tyrphostin AG 1478 (Sigma) diluted from stock in DMSO at doses varying from 5 to 12.5 μM in 8-well plates. AG1478 was used at 5 μM, 7.5 μM or 10 μM in most experiments. Embryos were cultured in the presence of AG1478 until the 7th cleavage stage (about 5 h post-fertilization), washed three times in ASW and cultured for 48 h (pluteus stage). In some experiments, in which localization of β-catenin or ERK was investigated, the embryos were allowed to develop with the compound until the 64-cell stage or mesenchyme blastula, respectively, in agreement with their temporal/spatial expression pattern. For TGF-α experiments 20 ng/ml TGF-α (SIGMA) was added together 10 μM AG1478 before first cleavage and the embryos were cultured for 48 h. The effect on morphogenesis was observed, depending on the experiment, at different times of development by microscopic inspection, and photographed using a Zeiss Axioscop2. To improve observation, in some cases the embryos were squashed and observed them differential interference (DIC) microscopy. In all experiments, embryos were also cultured in ASW or in ASW containing DMSO as controls, and assays were repeated three times with different batches.

Protein extraction and Western blotting

Total proteins were prepared by pulsed sonication for 90 s in solubilizing buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 2 mM PMSF, 10 μg/ml protease inhibitor, 1 mM Na_3VO_4, 1 mM NaF) from treated or control eggs and at different embryonic stages in ice. The lysates were centrifuged at 15,000*g* for 30 min at 4 °C and the supernatants used for Western blotting. Protein samples (50–100 μg) were electrophoretically separated using 4–20% gradient SDS–PAGE gels (NuPAGE, Invitrogen) and transferred onto nitrocellulose filters for immunoblotting. After blocking in 3% BSA in PBST, the Western blot was incubated with anti-EGFR primary antibodies (sc-120, Santa-Cruz Biotech.) (1:500), or anti-phosphotyrosine (clone 4G10, Upstate) (1:500), or anti-α-tubulin (clone B-5-1-2, Sigma) (1:2000) overnight at 4 °C. Primary antibodies were detected using the ECL chemiluminescence kit (Amersham) according to the manufacturer's instructions, and using secondary antibodies conjugated to horseradish peroxidase (1:1500) (Amersham).

Whole mount immunohistochemistry

Control and treated *P. lividus* embryos described above were collected at different times of development, depending on the experiments. The samples were fixed in 2% formaldehyde in sea water, permeabilized at −20 °C in methanol and rehydrated in PBS. Immunohistochemistry was carried out according to Romancino et al. [27]. For immunoreactions, the primary antibodies anti-EctoV (1:20), anti-Endo1 (1:20), anti-Ig8 (1:20), anti-H295 (1:5), β-catenin (gift of D. McClay) (1:500), anti-HE (gift of C. Gache) (1:200), anti-EGFR (1:25), and anti-dephosphorylated ERK1&2 (Sigma) (1:400) were incubated overnight at 4 °C. Secondary antibodies were: for anti-EctoV, anti-Ig8, anti-mouse Cy3-conjugate, for anti-Endo1, anti-mouse FITC-conjugate, for anti-β-catenin, anti-guinea pig FITC-conjugate, for anti-HE, anti-rabbit Cy3-conjugate (all from Jackson ImmunoResearch). Secondary antibodies described above were diluted 1:100 and incubated for about 1 h at room temperature. When anti-EctoV, anti-Ig8, anti-Endo1, anti-β-catenin, anti-HE were used, the secondary antibodies were anti-mouse Cy3-conjugate, anti-mouse FITC-conjugate, anti-guinea pig FITC-conjugate, anti-rabbit Cy3-conjugate (Jackson ImmunoResearch), respectively. The secondary antibodies described above were diluted 1:100 and incubated for about 1 h at room temperature. In order to detect EGFR, anti-ERK and H295 antigens, anti-mouse AP-conjugate (Sigma) (1:500) or anti-mouse HRP-conjugate (Sigma) (1:150) secondary antibodies were incubated for about 1 h at room temperature and then washed with PBST. Chromogenic detection was carried out in AP buffer (150 mM NaCl, 100 mM Tris–HCl pH 9.5, 25 mM $MgCl_2$, 5% polyvinylalcohol, 1 mM levamisole) with BCIP and NBT (Roche) or in DAB color reaction (10 ml Tris–HCl 50 mM pH 6.8, 5 mg Diaminobenzidine, 10 μl 30% H_2O_2). Finally, the eggs and embryos were resuspended in 80% glycerol in PBS and examined by microscopic inspection and photographed using a Zeiss Axioscop2 plus for DIC images. Epifluorescence was examined by microscopic inspection using a Leica DHL microscope and recorded photographically.

Results

EGFR is expressed during P. lividus development

The recently available draft of the genome sequence of *S. purpuratus* demonstrates that EGFR exists in the sea urchin as a single copy gene [28,29]. To ascertain the expression of the putative EGFR ortholog in *P. lividus* we extracted proteins from different developmental stages, separated them by SDS–PAGE and blotted them. The blot was incubated with human EGFR primary antibody. A band of the approximate size of known EGFR (180 kDa) was present throughout *P. lividus* development, suggesting that the antibody against the human EGFR specifically cross-reacts with a related *P. lividus* EGF receptor antigen, that we called EGFR-like (Fig. 1). We decided to analyse the spatial distribution of this antigen during sea urchin development (Fig. 2). Immunolocalization of EGFR-like reveals its presence both on the cell surface and in the cytoplasm of the egg (2A), and it maintains this ubiquitous presence in all the blastomeres until the 16-cell stage (2B). Instead, at the 32-cell stage, we found a reduced level of EGFR-like staining in micromeres (2C) and at the 64-cell stage it is absent in the small micromeres (2D). Moreover, we observed a large amount of this receptor in the parts of the cells directed towards the blastocoel cavity. At early blastula stage, the pattern of the stained cells resembled that of PMC precursors (2E and F). Staining of PMC continues to be detected during ingression at mesenchyme blastula and at this point it is possible to observe it on the basal side and in some apical ectodermal cells of the embryo (2G). At late gastrula stage, the cells at the bottom and at the top of the archenterons and the SMC are stained (2H). At pluteus stage, staining is observa-

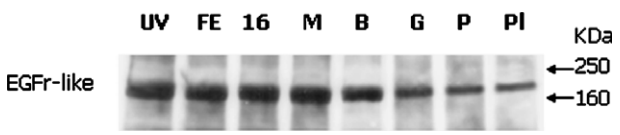

Fig. 1. Identification of an EGFR-like related antigen by Western blotting after SDS–PAGE analysis. Proteins extracted from *P. lividus* unfertilized (UV) and fertilized eggs (FE), 16-cell stage (16), morula (M) blastula (B), gastrula (G), prism (P) and pluteus (Pl) were blotted and the membrane was incubated with anti-EGFR (EGFR) human polyclonal antibody. The arrows on the right indicate molecular weights.

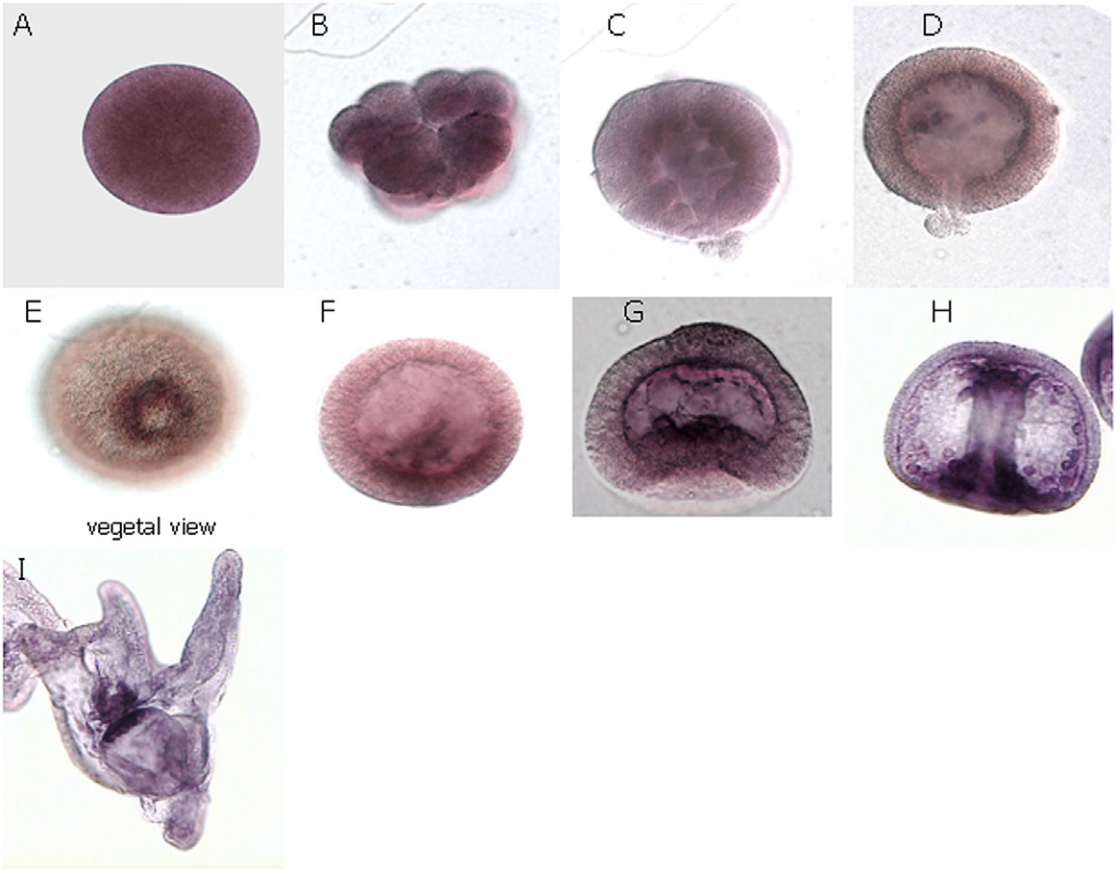

Fig. 2. Expression of EGFR-like during *P. lividus* development. (A–I) Whole mount immunolocalization using an anti-EGFR antibody. (A) Egg, (B) 16-cell stage, (C) 32-cell stage, (D) 64-cell stage, (E) early blastula vegetal view, (F) early blastula, (G) mesenchyme blastula, (H) late gastrula, (I) pluteus.

ble only in the section of the gut prior to the opening of the mouth (2I). These observations suggest that EGFR-like could play a role in the transmission of signals for the specification of determinate cell types.

Inhibition of EGFR-like receptor provokes changes in P. lividus development

In order to obtain some information about the role of the EGF receptor in *P. lividus* developmental events, we used AG1478, a tyrosine kinase inhibitor of the tyrphostin class that selectively blocks autophosphorylation of EGFR [26]. Autophosphorylation of receptors, following growth factor binding, creates specific phosphorylated tyrosine residues that serve as docking areas for downstream activated signal transducers, thus it is the starting point for a cascade of events. In order to examine whether a compound such as AG1478 has any effect on the phosphorylation of EGFR-like protein, fertilized *P. lividus* eggs were incubated with different concentrations of AG1478. Proteins extracted after 40 min of incubation were separated by SDS–PAGE and blotted. After incubation with anti-phosphotyrosine antibodies we observed a decrease in the phosphorylation of a band of 180 kDa inversely proportional to the increase in the drug concentration (Fig. 3). The same blot was incubated with anti-EGFR and we observed that the bands coincided with the previous (data not shown). The signal was quantified by comparison with tubulin after scanning and densitometry of the signal obtained following immunoblotting (data not shown).

In order to investigate when and where EGFR signalling is required, we analyzed the effect of blocking this signalling pathway on *P. lividus* early development. Fertilized eggs were incubated with

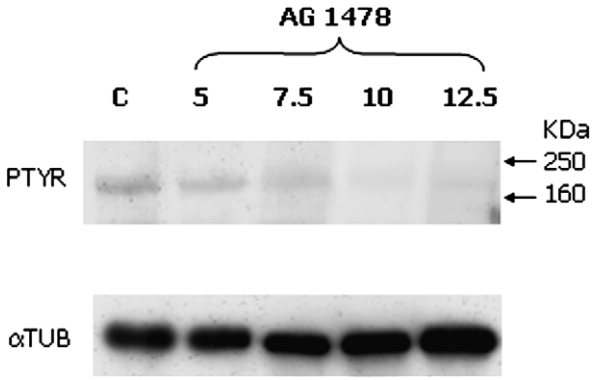

Fig. 3. Effect of AG1478 on EGFR-like phosphorylation. *P. lividus* fertilized eggs were incubated with 5 μM (5), 7.5 μM (7.5), 10 μM (10), 12.5 μM (12.5), AG1478 or without AG1478 (C). After 40 min of incubation, proteins were extracted from the different samples and after SDS–PAGE the blot was incubated with anti-phosphotyrosine (PTYR). Molecular weights indicated on the right. The blot was normalized using anti-α-tubulin (α-TUB).

different concentrations of AG1478 varying from 5 to 12.5 μM up to the 7th cleavage stage, then the embryos were washed and allowed to develop until control embryos, with or without DMSO, had reached the pluteus stage (Fig. 4A). Depending on the dose concentration, the embryos showed different morphologies with respect to controls (Fig. 4B–D). At lower drug concentrations the majority of the embryos showed no visible effects on development, and only a few showed an enlarged oral ectoderm. At higher concentration of AG1478, embryos displayed a range of phenotypes. At 7.5 μM, about 47% of the embryos arrested at the mid gastrula stage, at 10 μM

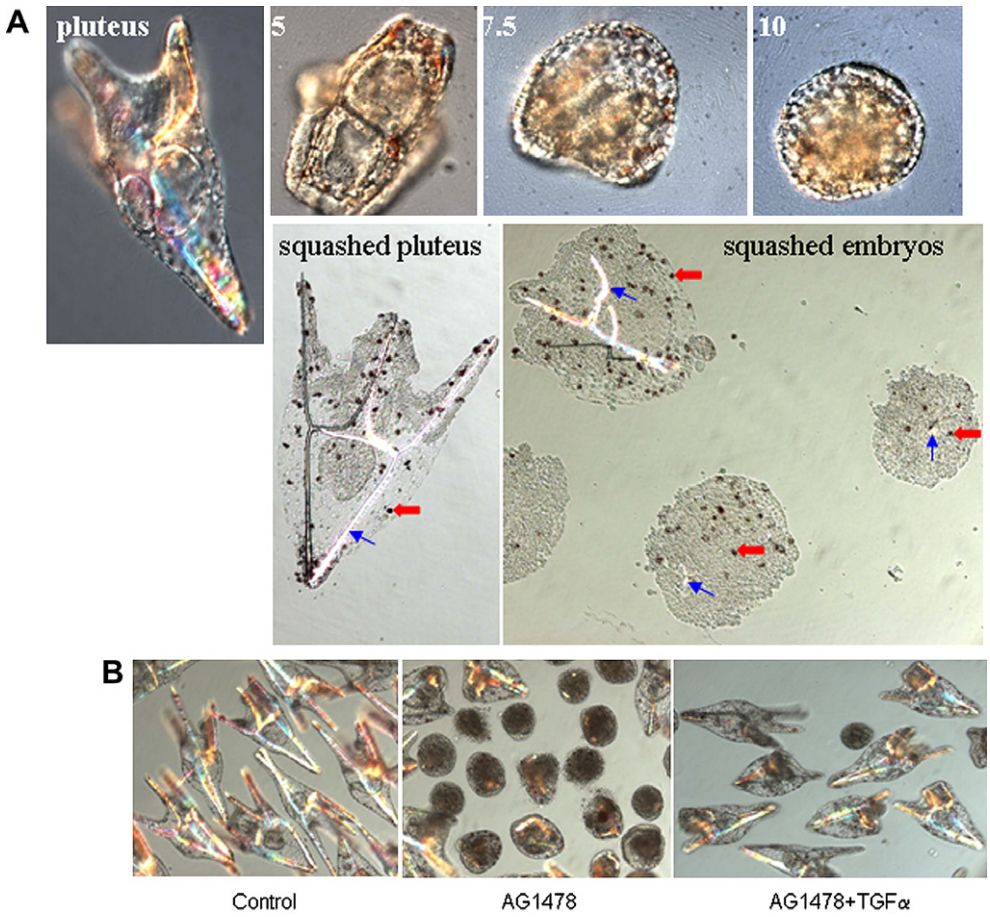

Fig. 4. *Paracentrotus lividus* embryos are sensitive to AG1478 treatment in a dose-dependent manner. (A) Altered morphology of embryos cultured in the presence of 5 μM (5 μM), 7.5 μM (7.5 μM), 10 μM (10 μM), AG1478 until the 7th cleavage stage and allowed to develop to 48 h. Control embryos cultured for 48 h until pluteus stage (control). Representative embryos from a range of similar phenotypes are shown in all the pictures. Pluteus (pluteus squashed) and embryos (embryos squashed) treated with AG1478 squashed on the slide (embryos squashed). (B) Embryos untreated (control) or treated with AG1478 (AG1478) or with AG1478 together TGF-α (AG1478–TGF-α). The blue arrows indicate the spicules, the red arrows indicate the pigmented cells.

about 20% arrested at mid gastrula and 70% were occluded blastula. These embryos lacked archenterons, primary mesenchyme-derived spicules were very small, abnormal or absent, secondary mesenchyme-derived pigment cells were in a decreased number. Moreover, these embryos had a cuboidal epithelium with extended cilia and resembled the features of animalized embryos. At 12.5 μM the embryos were degenerated. All data are summarized in Table 1. To better highlight the alteration on spicules and pigment cells the embryos were observed after squashing. Three different phenotypes are represented in Fig. 4. Spicules are smaller or absent, and the number of pigment cells progressively lower with respect to the controls, suggesting that EGFR-like signalling is required before the 7th cleavage stage and for the cell lineage of the large micromeres. Furthermore, to have more evidence that the drug inhibits signalling starting from EGFR-like antigen, we incubated embryos before first cleavage with both AG1478 and TGF-α, a canonical ligand of EGFR, and already utilized for similar experiments by other authors [25,30]. As shown in Fig. 4B, TGF-α has been able to rescue AG1748 disrupted/developmental arrested embryos and to recover also spiculogenesis. The obtained result suggests that TGF-α has antagonised the drug effect.

Table 1
AG1478 produces altered *P. lividus* phenotypes.

Morphology	Treatments (%)[a]				
	Control[b]	5 μM Tyrphostin AG1478	7.5 μM Tyrphostin AG1478	10 μM Tyrphostin AG1478	12.5 μM Tyrphostin AG1478
Normal plutei	98	76	46	0.5	
Occluded small plutei[c]	1	13	23	15	100 M.B. arrested and degenerated
Occluded prisms[d]	0	7	16	29	
Occluded blastula[e]	1	4	15	55.5	

[a] *P. lividus* grown in the AG1478 at indicated concentration until 7th cleavage after washed three times and cultured for 2–3 days at 20 °C. The results were taken from three different duplicate experiments.
[b] An equal amount of DMSO as highest concentration of AG1478 was added to control embryos.
[c] Small plutei with blastocoels filled with some cells and large oral hood.
[d] Small prims with blastocoels filled with some cells, small gut that never touch the archenteron roof and oral hood was absent.
[e] Blastulae filled with cells and without gut and oral hood.

Alteration of tissue-specific marker expression after incubation with AG1478

In order to investigate which territories have been altered after incubation of the embryos with the different concentrations of AG1478, we examined these embryos with lineage-specific markers (Fig. 5). We utilized both EctoV (5A) and UH295 (5D) as oral ectoderm markers [31], Ig8 as a primary mesenchyme cell marker

(5G) [32], and Endo1 as an endodermal marker (5L) [33]. As described previously, the embryos failed to complete morphogenesis and, depending on drug concentration, showed progressively decreased formation of vegetal derived structures. According to this result the Ig8 mesenchyme cell (5H and I) and the Endo1 endoderm cell (5M and N) markers, detected at 24 and 48 h of development respectively, showed decreased expression as the concentration of AG1478 at which they were incubated increased. Instead, EctoV, was expressed both in the thick epithelium, corresponding to oral ectoderm, and in other cells inside and throughout the surface of the embryoids (5B and C). The same result was obtained when we utilized anti-UH295, an antibody that specifically recognizes an epitope in ciliary band of the thickened epithelium (5E and F). These data support our previous morphological observations and indicate that by blocking EGFR signalling, the embryos develop an animalized phenotype with defects in A/V axis formation.

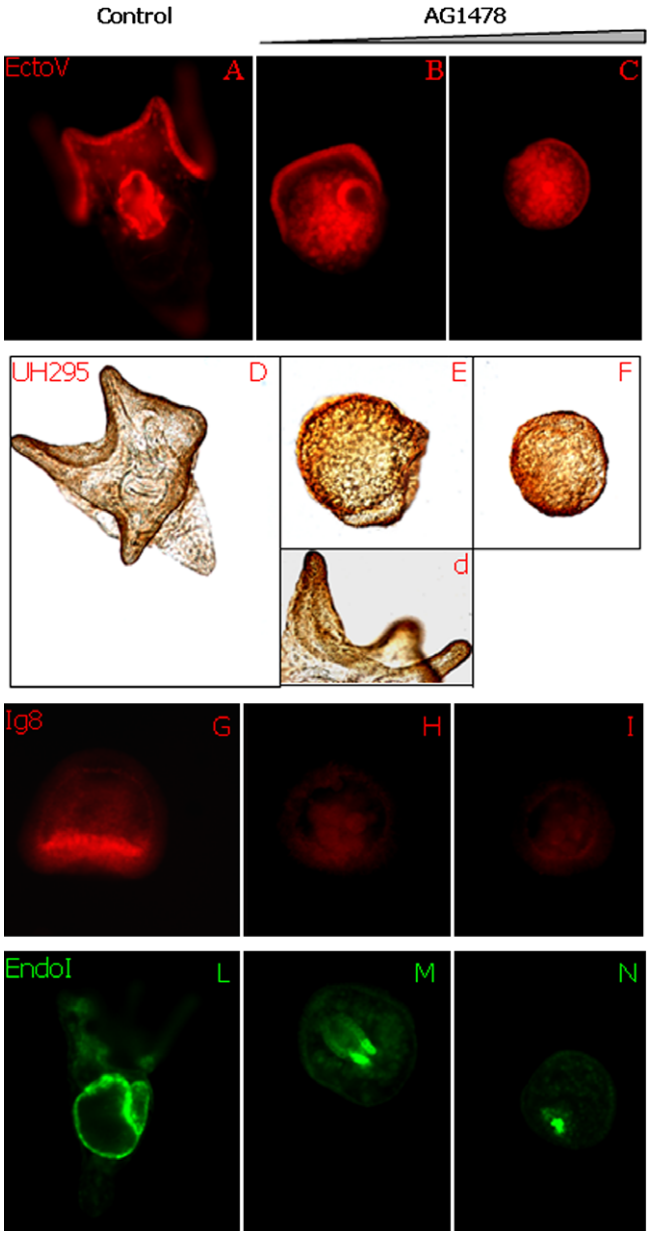

Fig. 5. Immunolocalization of tissue lineage markers after treatment with AG1478. Control embryos at 48 h of development incubated with anti-EctoV (A), anti-UH295 (D), anti-EndoI (L) or at 24 h of development incubated with anti-Ig8 (G). *P. lividus* embryos cultured with increasing amounts (5, 7.5, 10 μM) of AG1478 up to the 7th cleavage stage and allowed to developed to 48 h and incubated with anti-EctoV (B and C), anti-UH295 (E and F), anti-EndoI (M and N) or allowed to develop to 24 h and incubated with anti-Ig8 (H and I). (d) A detail of UH295 localization.

HE expression in embryos treated with AG1478

The hatching enzyme gene (HE) is a one of the earliest known genes activated following *P. lividus* fertilization and is expressed transiently during cleavage [34]. HE is normally expressed in a region corresponding to the presumptive ectoderm. By chemical treatment or experimental manipulation of specific genes it enlarge or restrict its localization along the A/V axis making it a useful early marker in the identification of vegetalized or animalized phenotypes [35–37]. Hence, in order to support our morphological observations described previously, and to investigate the possibility that EGFR-like signalling is required in gene expression of A/V determinants during early development, we monitored the spatial expression of HE in embryos treated with increasing amounts of AG1478 and cultured them until control embryos reached the hatching blastula stage (Fig. 6). Depending on the doses used, the size of the territory in which the HE protein is detected increases with respect to the control embryos (6B–D and D′). This effect is concentration dependent, thus, when the concentration of the compound increases a greater number of cells express HE indicating that the ectoderm/endoderm border along the A/V axis has been altered.

Effect of EGFR on β-catenin nuclearisation

During early stages, sea urchin embryos display a graded distribution of nuclear β-catenin along the A/V axis, being highest in the most vegetal blastomeres [38]. Nuclear β-catenin acts as a transcription co-activator with Lef1/Tcf1 and it plays a relevant role is in the vegetal signalling mechanism (VSM) and endomesoderm formation [39,40].

To examine whether EGFR-like signalling can be involved in the regulation of β-catenin entering into vegetal nuclei of early cleavage stage blastomeres, we examined the spatial distribution of β-catenin following AG1478 treatment. At the 64-cell stage, control embryos show a high amount of β-catenin in the nuclei of small and large micromeres and in *veg2* cells, and a progressive decrease from *veg1* cell to upper tiers. Instead, in AG1478-treated embryos, a reduced level of β-catenin was observed in the nuclei of micromeres and *veg2* cells, in a dose dependence manner (Fig. 7). These results suggest that β-catenin nuclearisation requires a functional EGF receptor-like and this is in agreement with the reduced endomesoderm in the AG1478-treated embryos (Figs. 4 and 5), and the enlarged HE pattern shown before (Fig. 6).

The EGFR–ERK signalling pathway is involved in early specification

One of the roles of EGFR in several systems is, upon binding to its ligand, to activate the ERK by phosphorylation. ERKs are serine–

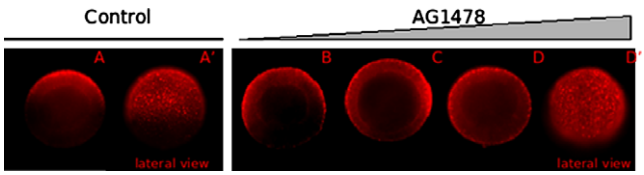

Fig. 6. AG1478 alters HE expression along A/V axis. *P. lividus* embryos cultured with 5 μM (B), 7.5 μM (C), 10 μM (D and D′) of AG1478 at hatching blastula or untreated (A and A′) incubated with anti-HE. Lateral view is indicated.

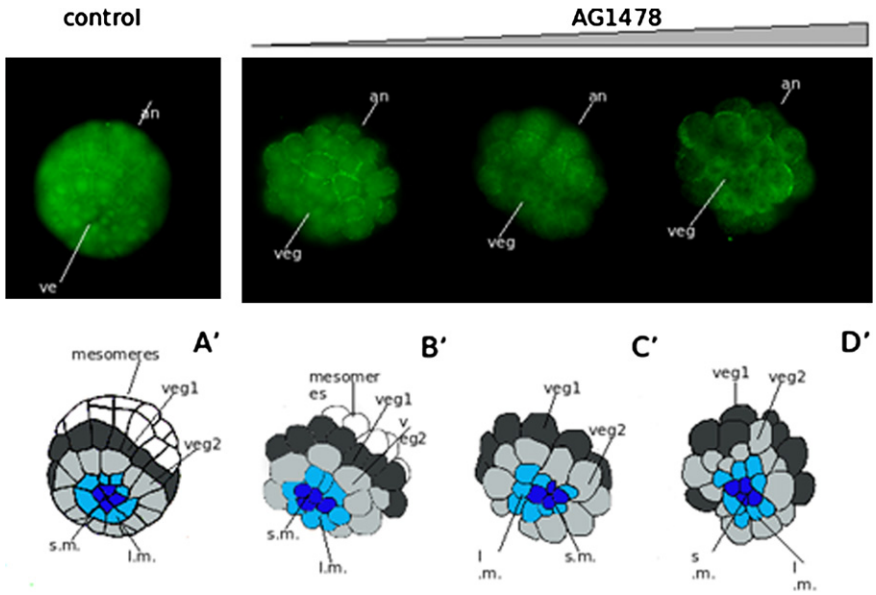

Fig. 7. EGFR-like signalling is necessary for β-catenin nuclearisation. Whole mount immunofluorescence of embryos at the 64-cell stage treated with of 5 μM (B), 7.5 μM (C), 10 μM (D) of AG1478 or untreated (A). Animal pole (an), vegetal pole (veg); a schematic representation of the 64-cell stage is shown in A′, B′, C′, D′; small micromeres (s.m.), large micromeres (l.m.), vegetative 1 (veg1), vegetative 2 (veg2), and mesomere tiers are indicated by the lines.

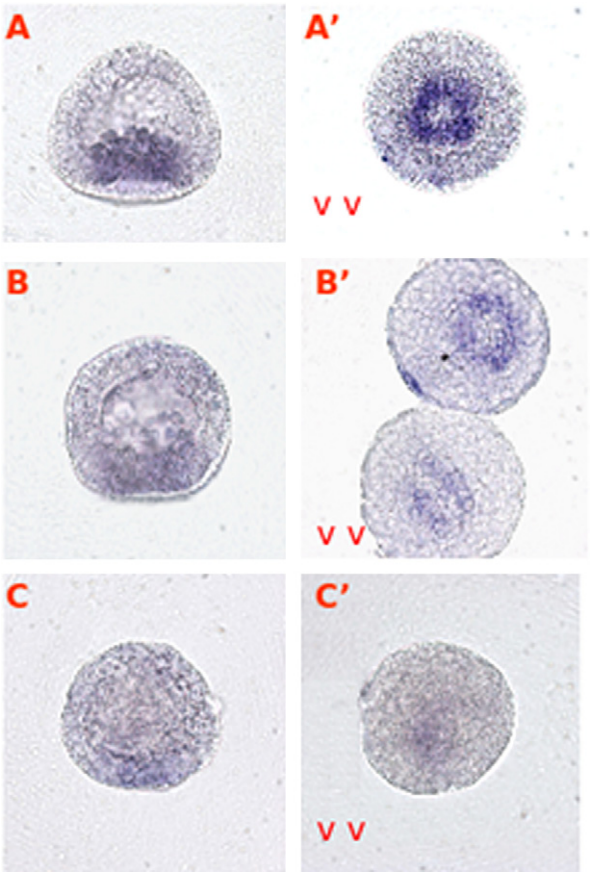

Fig. 8. EGFR-like signalling effects ERK expression. Immunohistochemistry with anti-ERK at mesenchyme blastula (A and A′), following AG1478 treatment up to the 7th cleavage stage (B and B′) and up to mesenchyme blastula (C and C′). The embryos are viewed from the side with the animal pole on top unless otherwise mentioned: vv, vegetal view.

threonine protein kinases that can phosphorylate substrates in different sub-cellular compartments and, in the nucleus, can activate specific target genes. The presence of an ERK-like antigen was demonstrated in *P. lividus* until second cleavage stage [41]. Further, it has been demonstrated that a diphosphorylated form of ERK has a restricted temporal and spatial pattern [42]. It is highly enriched in PMC precursors at blastula stage and is present until hatching, a pattern that resembled that shown in Fig. 2 for EGFR. Moreover, ERK functionality is necessary for micromere ingression, skeletogenesis and SMC cell fate. In order to establish whether EGFR is required for ERK activation we investigated the expression of ERK after AG1478 treatment using an antibody against the phosphorylated form of ERK.

Fertilized eggs were incubated with AG178 and a portion was washed at the 7th cleavage stage and left to develop until the mesenchyme blastula stage, another portion was incubated with the compound until the mesenchyme blastula stage. Treated embryos were fixed and stained with the antibody against the activated ERK (Fig. 8). When the embryos washed at the 7th cleavage stage were analyzed (8B and B′) the pattern of the stained cells was lower than in the controls (8A and A′). Instead, the embryos left to develop with AG1478 until mesenchyme blastula stage showed a large reduction in ERK staining in the PMC precursors (8C and C′) suggesting that inhibition of EGFR-like strongly prevents its activation.

Discussion

The epidermal growth factor receptor pathway plays a pivotal role in cell–cell communication and cell proliferation in both vertebrate and invertebrate. Through perturbation experiments, we have shown that an EGFR-like signalling pathway is required during *P. lividus* embryogenesis. In absence of this signalling, the embryo develops with many defects in the endomesoderm lineage and in extreme cases shows an animalized phenotype. Furthermore, these results are supported by the evidence that EGFR regulates both the presence of β-catenin in nuclei of the vegetal part and is responsible for the activation of a related ERK, two types of molecules that are important for the specification of the micromere–PMC lineage.

By spatial and temporal localization of EGFR-like we detected no specific distribution throughout cleavage, later in development,

instead, localization is restricted to a small number of cells in specific territories. This kind of localization suggested that EGFR-like activity could be required for at least two developmental programs: to regulate the gene expression of maternal determinants along the A/V axis, and to determine the specification of large micromeres and consequently correct endomesoderm differentiation. It seems that EGFR-like activated by specific ligands, one of these could be TGF-α, could give the input for specific gene network starting. This hypothesis was tested by perturbing embryo development until the 7th cleavage stage with AG1478, a compound that, as shown, selectively interferes with EGFR phosphorylation in a dose-dependent manner. Embryos, left to develop for 48 h, showed morphological defects ranging from mid gastrula to the most extreme animalized phenotype and in every case with some problems in gastrulation, spiculogenesis and the production of pigment cells. Moreover, the extreme animalizing effect after AG1748 treatment is in agreement with the opposite vegetalizing effect obtained when EGIP peptides or SfEGF-II, molecules containing EGF domains, were added to different sea urchin embryo species [43,44]. The altered morphology was rescued by TGF-α treatment. TGF-α has been successfully utilized in rescue experiments in which PDGFR and EGFR antagonists were employed [25,30]. Different possibilities have been proposed for rescue mechanism such as recovery of spiculogenesis and gastrulation by raising of second messengers levels, or by activation of an integrin signalling pathway [30]. Therefore, EGFR-like appears to be required for signalling before prehatching blastula. Additional evidence in relation to the effects of the inhibition of EGFR was obtained by immunohistochemistry with territorial markers. Indeed, we observed that the expression of Ig8 and Endo1, specific mesenchyme and endoderm markers, respectively, were reduced, whereas expression of EctoV and UH295 antigens, specific ectoderm and ciliary band markers, was unaffected. This is consistent with the observation that the so called apical ectoderm is autonomously specified and does not require any vegetal signals, in contrast to the oral ectoderm territory which is dependent on nuclear β-catenin [45–47].

The role played by EGFR-like in A/V axis formation was confirmed by monitoring HE gene expression. HE is normally expressed in an area corresponding to the presumptive ectoderm. At the 7th cleavage stage, inhibition of EGFR-like by various doses of AG1478 provokes an enlargement in HE expression territory, resulting in a progressive extension of the presumptive ectoderm territory and suggesting that the progeny of the blastomeres from the vegetal part have had their fates altered towards ectoderm. This is consistent with the fact that the compound can produce larvae with an animalized phenotype. It has been proposed that HE can be potentially expressed in all areas of the embryo but it is negatively controlled in the vegetal area [36]. The data here presented suggest that signals transduced from EGFR-like might activate a repressor that acts in the vegetal region of the embryo for this and other genes.

The activities of micromere progeny are regulated by β-catenin [36,48]. Localization of EGFR-like in micromere descendents and the morphological effects obtained following AG1478 treatment suggested that it could be responsible, through β-catenin, for the transmission of the signal necessary in these cells and to induce signals originating from such cells. At the 7th cleavage stage, treatment of the embryos with AG1748 results in a decrease in β-catenin in the nuclei of derived micromeres. We cannot exclude that an effect could be a decreased number of signals sent to veg2 cells with consequent failure of the process of endomesoderm specification. Furthermore, decreased β-catenin in nuclei at the 7th cleavage stage is in agreement with the enlarged expression of HE in embryos submitted to the same treatment [36,49].

A gene regulatory network (GRN) in which regulatory activator or repressor elements control spatial gene expression has been shown to exist for specification of sea urchin endoderm and mesoderm [21]. Presence of GRN seems to be a common requirement for gene regulation in all bilaterians that can use same regulatory genes for different purposes [50]. Such regulatory elements can respond to input affected by inter- or intra-cellular signals, and we cannot exclude that one or more of which might initiate from EGFR-like [51–53].

Different cell surface stimuli trigger ERK activation and modulate a large number of different cellular functions including proliferation, differentiation, survival, migration and adhesion. In sea urchins, ERK-mediating signalling is required for PMC ingression, spiculogenesis and mesoderm differentiation [42]. Furthermore, ERK signalling controls the epithelial–mesenchyme transition (ETM) of both primary and secondary mesenchyme cells [54]. One of the stimuli activating ERK we supposed came from EGFR signalling as demonstrated by experiments using AG1748 in which its presence is down-regulated and PMC ingression and mesoderm differentiation is affected. Furthermore, ERK activation depends on a functional TCF/β-catenin pathway [42] and here we have found that that is altered by AG1748 treatment.

ERK activity is important for the epithelial–mesenchymal transition (EMT) occurring during critical phases of embryonic development. During this phase mesenchyme cells acquire a morphology that is appropriate for migration in an extracellular environment. This process permits gastrulation in most metazoans. Many parallels are being found between this process of embryonic development and tumors [55]. Deregulation of ERK activity, indeed, contributes to uncontrolled cell proliferation and neoplastic progression. The results reported here clearly demonstrate that *P. lividus* EMT is inhibited when both EGFR-like and ERK activities are blocked. Hence, sea urchins constitute a good model system for obtaining information in relation to gene networking and the cell signalling involved or altered, even in human pathology. A molecular understanding of the pathways that control the development of a given tissue or cell type will also provide the basis for developing better combination therapies targeting different key components of the EGFR signalling network in the respective cancerous cells.

Acknowledgments

We thank Dr. Dave McClay for kindly providing antibodies against EctoV, UH295, Ig8, Endo1 and β-catenin. Dr. Christian Gache for the generous gift of the HE antibodies. We thank Dr. Sandy Chisholm for the revision of the English form of the manuscript and Mr. Alessandro Pensato for technical assistance.

References

[1] R. Schweitzer, B.Z. Shilo, Trends Genet. 13 (1997) 191–196.
[2] N. Moghal, P.W. Sternberg, Curr. Opin. Cell Biol. 11 (1999) 190–196.
[3] L.A. Nilson, T. Schupbach, Curr. Top. Dev. Biol. 44 (1999) 203–243.
[4] Y. Yarden, B.Z. Shilo, Cell 131 (2007) 1018.
[5] P.O. Hackel, E. Zwick, N. Prenzel, A. Ullrich, Curr. Opin. Cell Biol. 2 (1999) 184–189.
[6] J. Schlessinger, Cell 110 (2002) 669–672.
[7] D.J. Riese, D.F. Stern, Bioessays 20 (1998) 41–48.
[8] M. Sibilia, R. Kroismayr, B.M. Lichtenberger, A. Natarajan, M. Hecking, M. Holcmann, Differentiation 75 (2007) 770–787.
[9] R.P. Ray, T. Schupbach, Genes Dev. 10 (1996) 1711–1723.
[10] C. Van Buskirk, T. Schupbach, Trends Cell Biol. 9 (1999) 1–4.
[11] B.Z. Shilo, Development 132 (2006) 4017–4027.
[12] B. Schnepp, G. Grumbling, T. Donaldson, A. Simcox, Genes Dev. 10 (1996) 2302–2313.
[13] A.A. Simcox, G. Grumbling, B. Schnepp, C. Bennington-Mathias, E. Hersperger, A. Shearn, Dev. Biol. 177 (1996) 475–489.
[14] M. Golembo, E. Raz, B.Z. Shilo, Development 122 (1996) 3363–3370.
[15] M. Nishimura, Y. Inoue, S. Hayashi, Development 134 (2007) 4273–4282.

[16] N. Moghal, P.W. Sternberg, Oncogene 22 (2003) 5471–5480.
[17] L.M. Miller, H.A. Hess, D.B. Doroquez, N.M. Andrews, Genetics 156 (2000) 1595–1602.
[18] A. Stetak, E.F. Hoier, A. Croce, G. Cassata, P.P. Di Fiore, A. Hajanal, EMBO J. 25 (2006) 2347–2357.
[19] C. Van Buskirk, P.W. Sternberg, Nat. Neurosci. 10 (2007) 1300–1307.
[20] L.M. Angerer, R.C. Angerer, Curr. Top. Dev. Biol. 53 (2003) 159–198.
[21] E.H. Davidson, J.P. Rast, P. Oliveri, A. Ransick, C. Calestani, C.H. Yuh, T. Minokawa, G. Amore, V. Hinman, C. Arenas-Mena, O. Otim, C.T. Brown, C.B. Livi, P.Y. Lee, R. Revilla, M.J. Schilstra, P.J. Clarke, A.G. Rust, Z. Pan, M.I. Arnone, L. Rowen, R.A. Cameron, D.R. McClay, L. Hood, H. Bolouri, Dev. Biol. 246 (2002) 162–190.
[22] D.A. Hursh, M.E. Andrews, R.A. Raff, Science 237 (1987) 1487–1490.
[23] M.G. Delgadillo-Reynoso, D.R. Rollo, D.A. Hursh, R.A. Raff, D.R. Rollo, D.R. Hursh, R.A. Raff, J. Mol. Evol. 29 (1989) 314–327.
[24] J.E. Grimwade, M.L. Gagnon, Q. Yang, R.C. Angerer, L. Angerer, Dev. Biol. 14 (1991) 344–357.
[25] R.K. Ramachandran, V. Govindarajan, C.A. Seid, S. Patil, C.R. Tomlinson, Dev. Dyn. 204 (1995) 77–88.
[26] A. Levitzki, A. Gazit, Science 267 (1995) 1782–1788.
[27] D.P. Romancino, G. Montana, M. Di Carlo, Exp. Cell Res. 238 (1998) 101–109.
[28] E. Sodergren, G.M. Weinstock, E.H. Davidson, et al., Science 314 (2006) 941–952.
[29] F. Lapraz, E. Röttinger, V. Duboc, R. Range, L. Duloquin, K. Walton, S.Y. Wu, C. Bradham, M.A. Loza, T. Hibino, et al., Dev. Biol. 300 (2006) 132–152.
[30] R.K. Ramachandran, V. Govindarajan, C.A. Seid, C.R. Tomlinson, Mech. Dev. 44 (1993) 33–40.
[31] C.A. Ettenshon, D.R. McClay, Dev. Biol. 125 (1988) 369–409.
[32] D.R. McClay, G.W. Cannon, G.M. Wessel, R.D. Fink, R.B. Marchase, in: Jefferies, Raff (Eds.), "Time, Space and Pattern in Embryonic Development", Liss, New York, 1983.
[33] G.M. Wessel, D.R. McClay, Dev. Biol. 111 (1985) 451–463.
[34] T. Lepage, C. Gache, EMBO J. 9 (1990) 3003–3012.
[35] C. Ghiglione, G. Lhomond, T. Lepage, C. Gache, EMBO J. 12 (1993) 87–96.
[36] F. Emily-Fenouil, C. Ghiglione, G. Lhomond, T. Lepage, C. Gache, Development 125 (1998) 2489–2498.
[37] D.P. Romancino, G. Montana, S. Dalmazio, M. Di Carlo, Dev. Biol. 234 (2001) 107–119.
[38] C.Y. Logan, J.R. Miller, M.J. Ferkowicz, D.R. McClay, Development 126 (1999) 345–357.
[39] A. Vonica, W. Weng, B.M. Gumbiner, J.M. Venuti, Dev. Biol. 217 (2000) 230–243.
[40] L.M. Angerer, R.C. Angerer, Dev. Biol. 218 (2000) 1–12.
[41] S. Chiri, C. De Nadai, B. Ciapa, J. Cell Sci. 111 (1998) 2519–2527.
[42] E. Rottinger, L. Besnardeau, T. Lepage, Development 131 (2004) 1075–1087.
[43] T. Suyemitsu, T. Asami-Yoshizumi, S. Noguchi, Y. Tonegawa, K. Ishihara, Cell Differ. Dev. 26 (1989) 53–66.
[44] N. Kamei, W.J. Swanson, C.G. Glabe, Dev. Biol. 225 (2000) 267–276.
[45] A.H. Wikramanayake, W.H. Klein, Development 124 (1997) 13–20.
[46] S. Yaguchi, J. Yaguchi, R.D. Burke, Development 133 (2006) 2337–2346.
[47] S. Yaguchi, J. Yaguchi, R.D. Burke, Dev. Biol. 302 (2007) 494–503.
[48] A.H. Wikramanayake, L. Huangand, W.H. Klein, Proc. Natl. Acad. Sci. USA 95 (1998) 9343–9348.
[49] T. Lepage, C. Sardet, C. Gache, Dev. Biol. 150 (1992) 23–32.
[50] E.H. Davidson, D.H. Erwin, Science 311 (2006) 796–800.
[51] E.W. Howard, L.A. Newman, D.W. Oleksyn, R.C. Angerer, L.M. Angerer, Development 128 (2001) 365–375.
[52] A.P. Kenny, D.W. Oleksyn, L.A. Newman, R.C. Angerer, L.M. Angerer, Dev. Biol. 261 (2003) 412–425.
[53] P. Oliveri, K.D. Walton, E.H. Davidson, D.R. McClay, Development 133 (2006) 4173–4181.
[54] M. Fernandez-Serra, C. Consales, A. Livigni, M.I. Arnone, Dev. Biol. 268 (2004) 384–402.
[55] J.P. Thiery, Nat. Rev. Cancer 2 (2002) 442–454.