The influence of cell interactions and tissue mass on differentiation of sea urchin mesomeres*

ODED KHANER† and FRED WILT

Department of Molecular and Cell Biology, 371 LSA University of California, Berkeley, CA 94720, USA

* Supported by NIH grant HD-15043.
† Supported by NIH grant TW-04241.

Summary

The developmental potential of different blastomeres of the sea urchin embryo was re-examined. We have employed a new method to isolate substantial numbers of different kinds of blastomeres from 16-cell-stage embryos, and we have used newly available molecular markers to analyze possible vegetal differentiation. We have found that, while isolated mesomere pairs behave according to the classical expectations and develop into ectodermal vesicles, there is a clear effect of reaggregating two or more mesomere pairs. They survive better in long-term culture and, after prolonged periods, they display an astonishing ability to express vegetal differentiation. We also combined mesomeres with stained micromeres or macromeres from the vegetal hemisphere. Although induction of guts and spicules was observed, there was little if any effect of varying the ratio of different blastomeres on the kinds of differentiation obtained.

Key words: cell interaction, tissue mass, differentiation, sea urchin, mesomere, blastomere.

Introduction

When the sea urchin embryo was submitted to the classical operations of experimental embryology, great differences in the fates and potentialities of the different blastomeres were revealed, and interactions between different blastomeres caused spectacular changes in cell fate (Horstadius, 1939, 1973). The interpretations of these results have recently been reviewed extensively (Wilt, 1987; Davidson, 1989). This classical work has been important in the history of the concepts of developmental gradients and induction.

We wish to re-examine some key experimental findings because the ability to reliably mark cells with lineage tracers, and the ability to identify the differentiated state of cells by molecular criteria, may help to illuminate how determination occurs. For example, blastomeres taken from the animal hemisphere of early cleavage stage embryos usually form ciliated epithelial spheres with little obvious differentiation. Early studies had to rely on identification of differentiated cells by histological criteria, and hence one could not have known the extent of differential gene expression in these isolated animal hemispheres. Recent work has shown these isolates express several markers characteristic of their normal differentiated fate (Livingston and Wilt, 1989). Furthermore, if formation of differentiated tissue depends to some extent on normal morphogenetic movements, as the formation of the gut does, then identification of the outcome of a given manipulation may be very difficult if the manipulations interfered with morphogenesis.

We report here two kinds of experiments designed to evaluate the developmental potential of the mesomeres of the animal hemisphere. First, we ask if the differentiation of isolated mesomeres is in any way influenced by the number of blastomeres in the explant, the so-called 'mass effect' invoked by embryologists on many occasions. These experiments not only revealed an effect of mass, but showed a heretofore unsuspected ability of mesomeres to form gut, spicules and pigment cells after prolonged periods in culture. Second, we combined mesomeres with cells from the vegetal hemisphere (micromeres or macromeres) that had been stained with a lineage tracer. The ratio of the number of vegetal cells to the number of mesomeres was varied systematically. Although induction was observed, there was little if any effect of varying the ratio of cells on the kinds of differentiation obtained, a result different from those obtained by transplantation.

Materials and methods

Isolation and culture of blastomeres (Fig. 1)
Gametes of *S. purpuratus* and *L. pictus* were obtained and
1. Fertilization
2. 4 cell
3. 16 cell
4. Separate
5. Spin
6. Culture

Fig. 1. Method of blastomere isolation. The scheme shows the way embryos were dissociated at the 16-cell stage. Pairs of mesomeres, micromeres and macromeres were selected, and appropriate numbers of blastomeres centrifuged into aggregates of different sizes.

Eggs were fertilized in sea water (SW) containing 2 mM para-amino-benzoic acid (PABA). The fertilization membranes were removed by passing the embryos through 55 μm Nitex. Embryos were cultured by conventional methods (Hinegardner, 1967). At the second cleavage division, the embryos were resuspended in calcium-free sea water (CFSW), and cultured to the 16-cell stage. In most of the cultures, the majority of the blastomeres were gently dissociated into pairs of sister cells (mesomere-mesomere, macromere-micromere). In some cultures in which the blastomeres did not separate at the 16-cell stage, the embryos were centrifuged and resuspended in calcium-/magnesium-free sea water (CMFSW), until most of the blastomeres were dissociated into pairs. The dissociated cells were placed in a Petri dish that contained a layer of 1% agarose in CFSW. The different types of blastomeres, or pairs of blastomeres, were then isolated, using a micropipette and placed in another Petri dish until the fifth cleavage division. According to the type of experiment, descendants of these blastomeres were placed together in glass tubes with a sharply conical bottom containing SW with 2% horse serum, and centrifuged gently to bring them in contact. After 1 h the aggregates were removed by a mouth micropipette from the glass tubes, and centrifuged gently to form mesomere pairs, and centrifuged into aggregates of different sizes.

**Role of the third cleavage plane**

The third cleavage plane of the sea urchin embryo delineates the boundary between the animal and the vegetal halves of the embryo. Displacement of the third cleavage plane towards the vegetal pole occurs in some cultures, and results in animal blastomeres with a greatly increased tendency to form vegetal structures (Driesch, 1900; Henry et al., 1989). This is why the location of the third cleavage plane in these cultures is crucial to the success of the isolation of pure mesomeres. In the experiments reported here, only cultures that exhibited a truly equatorial third cleavage plane were used for blastomere isolation.

**Scoring morphological characteristic of the embryoids**

After three days, and during the succeeding four days in culture, embryoids were examined for the appearance of gut-like or invaginated structures, spicules and pigment cells. Gut-like and invaginated structures could be observed under the dissecting microscope. Embryoids were scored as positive if an organized hollow group of cells, or hollow tube, was present in the blastocoel cavity. Spicules could be identified in embryoids as fine pins. Some embryoids from every culture were flattened with a cover slip to facilitate visualization of the spicules under the compound microscope. Pigment cells were observed by their bright orange-red color, and were easily identified under the dissecting microscope.

**Alkaline phosphatase activity**

Embryoids cultured for four days were placed in a drop of SW on a poly-lysine-coated microscope slide and allowed to settle for ten minutes. A drop of fast blue RR salt (4-benzoylamo 2,5-dimethoxybenzenediazonium chloride hemi [zinc chloride] salt) in 2.5% (w/v) Naphthol AS-MX phosphate (Sigma) was added, and the slides were incubated for 15 min in a moist chamber, at 37°C. Embryoids cultured for four days were treated the same as above, and used as controls for the alkaline phosphatase staining.

**The monoclonal antibody ENDO I**

Embryoids cultured for four days were placed in a drop of SW on siliconized depression slides. The embryoids were fixed in cold methanol for 20 min and were rinsed twice with cold SW. The embryoids were then moved to poly-lysine-covered coverslips in a moist chamber. They were blocked in 1:1 normal goat serum (Vector): sea water, for 60 min at room temperature, and were then rinsed once with SW. Incubation with the monoclonal antibody Endo I was carried out at room temperature for 60 min. (The monoclonal antibody Endo I, was kindly donated by David McClay). The embryoids were rinsed twice with cold SW, and once with cold phosphate-buffered salt solution (PBS) and then stained with antimouse polyclonal immunoglobulin conjugated with fluoroescein isothiocyanate (FITC) (Sigma) 1:20 in PBS, for 2 h at room temperature. Finally, the embryoids were rinsed four times with PBS and mounted with 20 μl of mounting media containing 70% glycerol, 30% water and 0.5% PABA (Wessel and McClay, 1985).

**RITC staining**

Cultures of *L. pictus* were separated at the 8-cell stage and placed in CFSW. One half of the culture was used to isolate mesomere pairs at the 16-cell stage. The other half was placed in a beaker containing rhodamine B isothiocyanate (RITC) in CFSW, made up as described by Ettensohn and McClay (1986). At the 16-cell stage, the embryos were rinsed in CFSW until most of them dissociated to single cell suspension. RITC-stained macromeres or micromeres were placed in the same glass tube with unstained mesomere pairs, and centrifuged together.

**Probe preparation**

The SM-50 probe is derived from 1.3 kb sequence that was isolated and characterized by Sucov et al. (1987) and Benson et al. (1987), and subcloned into the pSP65 transcription vector. SP6 polymerase (Promega biotech) and [3H]-UTP were used to synthesize a [3H]-labeled single-stranded RNA probe (Melton et al. 1984).
In situ hybridization
Embryoids were placed in a drop of SW in a siliconized depression slide, and were fixed in glutaraldehyde by the method of Cox et al. (1984). The embryoids were dehydrated, stained with eosin, embedded in paraffin in a depression slide under a dissecting microscope mounted on a warm plate, sectioned (5μm) and mounted on poly-lysine-coated microscopic slide. The protease treatment, hybridization steps, RNase A digestion and autoradiography were all performed according to Cox et al. (1984). NTB-2 emulsion-coated slides were exposed at 4°C for 6 weeks before developing.

Results

Differentiation of isolated mesomeres
We wished to vary the number of mesomeres in an animal hemisphere dissociated into pairs, to see if there is some effect of the number of blastomeres in the original isolate on the outcome of the experiment. If there is, this would indicate some interactions between animal hemisphere cells that influence their development. Although isolated animal hemispheres have been cultured by many experimenters, and occasionally two hemispheres fused, there is no systematic examination of this point in the literature.

We have recently developed a method to isolate fairly large numbers of different kinds of blastomeres from the 16-cell stage by dissociation of the embryo into mesomere pairs and macromere–micromere pairs. This method has been applied to obtain large numbers of mesomeres, which are then reaggregated into explants of defined size by gentle centrifugation, followed by long-term culture in sea water. Livingston and Wilt (1989) have previously shown that if embryos are used only from eggs that undergo strictly equatorial cleavage, there is no systematic examination of this point in the literature.

Fig. 2 shows a graphical representation of the survival of these embryoids of different size aggregates (2, 4, 8 and 16) of mesomeres, after 3 and 6 days, of both species, S. purpuratus and L. pictus.

Embryoids were examined for staining by the monoclonal antibody Endo I and histochemical tests for alkaline phosphatase. Embryoids that were not scored on morphological grounds as positive did not display these markers of gut differentiation, while all those scored positive that were examined did so. The appearance of the alkaline phosphatase enzyme activity was dependent on the extent of the gut, so large or more developed guts stained much more darkly than small gut-like structures (Fig. 5). Embryoids surviving past three days also showed spicules, and embryoids were examined both with transmitted light and polarized light to confirm their presence. There is a marked difference in the propensity of the survivors in the two species to form spicules, the percentage being much higher in S. purpuratus, where 50–60% of the survivors showed spicules, and these were usually evident by 96h. We were astonished to see that embryoid cultures from these mesomere reaggregates began to show clear evidence of vegetal differentiation, forming guts, spicules and pigment cells, in embryoids derived from 4 or more mesomeres after prolonged time in culture (Figs 3 and 4). In both species, up through 60h, the embryoids did not display any indication of archenteron formation, or of mesenchyme aggregates and spicule formation, or of pigmented epithelial cells. This is the result reported scores of times by hosts of workers since the time of Driesch and Boveri (Wilson, 1925).

We were astonished to see that embryoid cultures...
Fig. 3. Morphology of mesomere development. The photos (A, B, and C) show the appearance of embryoids derived from 4 mesomeres of *S. purpuratus* cultured for 3 days (A, x100), 5 days (B, x200), and 7 days (C, x400). Photos (D, E) show embryoids of *L. pictus* that were developed from mesomere pair after 5 days (D, x400), and of embryoid from 8 mesomeres after 7 days (E, x200). G = gut, s = spicule, P = pigment cell, and C = cilia.

...go on to form spicules, though this has not been explicitly shown. These embryoids showed a few scattered cells with many grains against a rather high background, which is sometimes observed with this material even when intact embryos are examined. Cells labeled to this intensity are never observed in non-spiculegenic cells of the normal embryo or when control sense probes are used (Fig. 6).

Finally, though no pigment cells whatsoever are detectable by 72 h of culture for either species, clear examples of pigment cells occurred in about 25% of the survivors (after 72 h) in *L. pictus*, and about 70% in *S. purpuratus*.

The exception to the description above is the embryoids derived from a single pair of mesomeres. In *S. purpuratus*, such cultures do not survive past 72 h, and show almost no sign of differentiation of vegetal structures or of SM-50 expression (Livingston and Wilt, 1989). Even the 5% of these embryoids that did survive 96 h did not show any vegetal differentiation. However, in *L. pictus*, 193 of 502 explants of mesomere pairs survived 72 h, and 30% of these 193 were alive and swimming after 6 full days in culture. These embryoids formed only a very low percentage of gut-like structures, about the same seen after 3 days, and formed virtually no spicules or pigment cells. Nor did staining for alkaline phosphatase or monoclonal antibody Endo I reveal covert archenteron formation.

We conclude that there is a striking threshold effect of the number of mesomeres in the explant, which improves their survival in culture and, furthermore, that there is threshold for the ability of these animal hemisphere cells to form vegetal structures, like gut, spicules and pigment cells, after prolonged culture.
Cell interactions and differentiation of sea urchin mesomeres

The effect of number of mesomeres (S. purpuratus) on differentiation after 6 days

No. survived 3 days

238 116 291 246

The effect of number of mesomeres (L. pictus) on differentiation after 6 days

No. survived 3 days

193 66 68 56

**Fig. 4.** Differentiation of isolated mesomeres. The graph shows the differentiation of isolated mesomeres, of *S. purpuratus* and *L. pictus*, after 6 days culture.

**Differentiation of mesomeres co-cultured with micromeres**

The culture of different number of micromeres with mesomeres from the animal hemisphere may give rise to embryoids that are very near normal both in form and in the differentiation of the different tissue. This result is a corner stone of ideas about gradients of developmental potential and cell interactions in the sea urchin embryo. Therefore, we decided to examine this issue once again, using the large numbers of mesomeres available from the dissociation technique, and staining the micromeres with a lineage tracer so that derivatives of the two blastomere types could be identified. Since we have learned from the experiments described above on culture of mesomeres alone that the number of mesomeres, and the time in culture, are important to the outcome, we have systematically varied the numbers and ratios of cells, and have looked primarily at the differentiation attained by these cultures by 72 h in culture, or before. There is one very important difference, at least, between this experimental design and the

**Fig. 5.** Alkaline phosphatase Staining. (A,B). The appearance of the gut antigen ENDO 1: (C,D). The photos (A,B) show a positive staining for alkaline phosphatase enzyme activity, of *S. purpuratus* embryoids derived from: 4 mesomeres (*A* ×200) and 16 mesomeres (*B* ×200), after 4 days of culture. The photos (C,D) show a bright-field (*C* ×200) and fluorescent image (*D* ×200) of an embryoid derived from 8 mesomeres, after 4 days of culture. Even though a differentiated gut is difficult to see in the bright-field, the monoclonal antibody Endo 1 shows a clear localization.

**Fig. 6.** In situ hybridization of SM-50. The photos show in situ hybridization to 3-day-old embryoid, using ^3^H-labeled SM-50 probe. Bright field (*A* ×400) of an embryoid from 16 mesomeres shows 3 centers of positive cells (arrows).
Differentiation in meso: micro combinations (after 2 days)

<table>
<thead>
<tr>
<th>Ratio mesomeres: micromeres</th>
<th>% Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:0</td>
<td>90</td>
</tr>
<tr>
<td>8:0</td>
<td>80</td>
</tr>
<tr>
<td>16:0</td>
<td>70</td>
</tr>
<tr>
<td>16:2</td>
<td>60</td>
</tr>
</tbody>
</table>

Fig. 7. Differentiation of mesomeres cultured with micromere. Quantitative summary of differentiation obtained with mesomeres alone, and combinations of mesomeres with micromeres. When micromeres are present, gut, spicules and pigment cells arise within 48 h. As the ratio of the number of micromeres is increased, there is little if any effect. The survival of the embryoids up to 72 h tends to improve from 50% to 70%, as the aggregates become larger.

transplants carried out by Horstadius; the cells used here intermix, and coherent micromere quartets or mesomere octets were not employed. All these experiments were carried out with a single species, *L. pictus*, and we always employed four mesomeres or more in the aggregate, above the mass effect threshold for *L. pictus* revealed in the experiments described above. The survival up to 72 h was excellent in all instances, tending to improve from 50% up to 70% as the aggregates become larger. Fig. 7 summarizes the results of these experiments. Comparing the kind of differentiation obtained with or without micromeres present, the difference is striking and in line with previous results of others (Horstadius, 1935; Livingston and Wilt, 1989). When micromeres are present, gut, spicules and pigment cells arise. Invagination and gut-like structures are present within 48 h in 50% of the cases, spicules are present in about 60%, and pigment cells are noticeable in about 8-10% of the embryoids, a low percentage but greatly above that seen without any micromeres present. The gut-like structures that form stain for alkaline phosphatase and Endo I; when guts are not present, there is no staining. Examination of the embryoids at 3 days show that gut structures never contain the fluorescent cells derived from micromeres: the guts are truly induced. Likewise, spicules that are present are always surrounded by fluorescent marked cells. One cannot exclude the possibility that mesomere-derived cells could participate in spicule formation, but we do not see spicules without micromere derivatives surrounding the spicules (Fig. 8A,B). The pigment cells that form are all induced; they are not derived from micromeres. As the ratio of the number of micromeres to mesomeres is increased, there is little if any effect. Even the lowest number of micromeres is sufficient to induce gut in half of the embryoids, spicules in half of them, and pigment cells in 8%. Increasing the ratio of micromeres may increase the number of embryoids with spicules, but since spicules form autonomously from micromeres, this is not evidence for some profound inductive effect of micromeres on mesomeres.

Differentiation of mesomeres co-cultured with macromeres

Fig. 9 shows the results of culturing isolated mesomeres that are aggregated with lineage tracer marked macromeres, at two different ratios. Survival of cultures was 60-70% (surprising in view of the fact that in our experience macromeres cultured alone survive poorly in culture). Among the survivors, most formed authentic gut-like structures. Many of the gut structures that form when the macromere/mesomere ratio is lower are apparently just as developed as when the ratio is higher; though, when the number of mesomeres is relatively high, there is a tendency for there to be 2 gut-like structures (although, one case was observed with 3 guts) in the central cavity of the epithelial sphere (Fig. 8C,D). In all these instances, one gut was composed of labeled macromere-derived cells, and the other was composed exclusively of mesomere derivatives.

About 20% of the embryoids formed pigment cells that were derived from the macromeres, which are the normal source of pigment cells (Gibson and Burke, 1985). Only 10% of the embryoids formed spicules. These spicules were surrounded by stained cells, and are probably formed primarily by differentiation of secondary mesenchyme from macromeres which may then transdifferentiate to spiculogenic mesenchyme as described by Fukushi (1962), and Ettensohn and McClay (1988). There is no marked effect of macromere:mesomere ratio within the limited range examined in the experiments.
Fig. 8. Combinations of mesomeres with micromeres (A,B) and with macromeres (C,D). Photos show the differentiation of embryoids derived from combinations of mesomeres with micromeres or macromeres. Bright field (A ×200) shows embryoid derived from 8 mesomeres and 4 micromeres, and fluorescent field (B ×200) of the same embryoid. The spicules that are present are always surrounded by fluorescent marked cells. Bright field (C ×200) shows embryoid derived from 16 mesomeres and 4 macromeres. Multiple gut-like structures appear in the central cavity of the epithelial sphere. Fluorescent field (D ×200) shows that only one gut of all the gut-like structures present is surrounded by fluorescent cells. s=spicule g=gut.

Fig. 9. Differentiation of mesomeres cultured with macromeres. Differentiation obtained with mesomeres cultured alone, or with lineage tracer marked macromeres, at two different ratios. Most of the embryoids formed authentic gut-like structures. About 20% formed pigment cells derived from macromeres, and only 10% formed spicules. Survival of the cultures was 60% – 70%.
Discussion

Developmental potential of the mesomeres

The strategy for obtaining large numbers of different kinds of blastomeres from the 16-cell-stage embryo utilizes disaggregation into blastomere pairs in CFSW. Even though this is a mild regimen, and CFSW was used to 'loosen' blastomeres in classical experiments, the cell surface (and/or interior) may well have sustained alterations. However, a careful search for the effects of CFSW on mesomere differentiation did not reveal any effects of CFSW (Henry et al. 1989). While isolated mesomere pairs behave more or less according to classical expectations, there is a clear effect of reaggregating two or more mesomere pairs. They survive better in long-term culture and, after prolonged periods, long after normal differentiation and establishment of pattern has occurred in intact embryos, they display a clear ability to form many tissues of the embryo normally derived from different lineages of the vegetal hemisphere.

Recently Henry et al. (1989) have postulated that interactions among mesomeres may restrict their developmental potential during embryogenesis. This is based on two kinds of evidence: first, mesomeres obtained from different stages of the embryo, viz., 8-, 16- and 32-cell, showed a progressive decrease in ability to form vegetal structures within 72 h; and second, reaggregates of mesomeres and animal hemispheres from the 16-cell-stage embryo differentiate vegetal structures less extensively than do mesomeres pairs during the 72 h of culture. They observed that about 1/4 of both isolated mesomere pairs and aggregates of 8 mesomeres showed differentiation of vegetal characteristics within 3 days; this extent of differentiation is much greater than that observed here during the first 3 days in culture, but Henry et al. used mesomeres from embryos that displayed subequatorial, equatorial and supraequatorial 3rd cleavages, an important variable discussed before. When their experiments employed only equatorially cleaving zygotes, which are the only type used in all our experiments, only 9% of the embryoids showed gut formation, close to the 8% we observed. Similary low numbers were seen by Livingston and Wilt (1989, 1990). There is little difference in actual data when the same kinds of isolates are compared. Their conclusion that reaggregation restricts developmental potential of mesomeres is based on their expectation that if one pair of mesomeres shows vegetal characteristics 24% of the time, then aggregates of more than one pair of mesomeres should show much higher frequencies, an expectation that depends on the exact mechanisms by which vegetal characters develop. The fundamental difference in observations is that our conclusions of the effect of aggregation is based on prolonged survival and differentiation well past the 3 days, a time after the experiments of Henry et al. (1989) were terminated. Hence, insofar as direct comparison of results is possible, they are consistent. Our interpretation that aggregate size influences survival and differentiation is based on results obtained with cultures much older than those used in the study by Henry et al. (1989).

This delayed and atypical differentiation of mesomeres is certainly consistent with the plasticity of these cells in response to Li+, and clearly shows that there is extensive latent capacity of these cells to differentiate gut, spicules and pigment cells. It is also clear that there are homotypic interactions among these blastomeres that favor survival and enhance this latent capacity in long-term culture.

There are apparently interactions between mesomeres that may restrict the differentiation of vegetal characters during the first 3 days of development in culture; there are also interactions between blastomeres that favor extensive vegetal differentiation in long-term culture (more than 3 days), as shown here.

We may ask what is the meaning of this plasticity of mesomeres for understanding normal development. First, there is the possibility that during normal development there are mechanisms that suppress the latent ability of mesomeres to form such a large variety of tissues (Henry et al. 1989). Second, as the hoped for progress in understanding the mechanisms of cellular interactions in embryos occurs, models will have to reckon with the latent ability of mesomeres to form almost all the tissues of the larva. We propose that these latent abilities of mesomeres are governed by a delicately balanced regulatory network(s), and that over prolonged periods of time in culture the regulation directing normal mesomere fate breaks down.

Interactions of mesomeres

The results obtained with combinations of micromeres or macromeres with cells of the animal hemisphere seem at first glance what one could expect. In the combined cultures, there is a much wider range of differentiation than would occur in mesomeres alone under those conditions of culture and, hence, cell interactions must have occurred.

Micromeres evoke gut differentiation in mesomeres, differentiate themselves into spicules and stimulate a very low level of pigment cell appearance. Varying the number of micromeres with a given number of mesomeres does not change this basic response.

Macromeres also self-differentiate to form gut spicules and pigment cells when they are combined with mesomeres, and all these tissues would be expected to form from macromeres. Varying the number of mesomeres with a given number of macromeres does not change this basic response, but when a large number of mesomeres are present, there may be instances of multiple gut formation. The lineage tracing also indicates that mesomeres have been induced by macromeres to participate in gut formation in addition to the contribution of macromeres themselves to gut development. We wish to underline that the quantitation of the inductive response used here is all or none; there either is a gut, a spicule, a pigment cell, or there isn't. Since the inducing micromeres or macromeres are themselves able to form spicules, it is essentially the extent of gut formation from mesomeres that could be variable in
Cell interactions and differentiation of sea urchin mesomeres

Fig. 8. Combinations of mesomeres with micromeres (A,B) and with macromeres (C,D). Photos show the differentiation of embryoids derived from combinations of mesomeres with micromeres or macromeres. Bright field (A x200) shows embryoid derived from 8 mesomeres and 4 micromeres, and fluorescent field (B x200) of the same embryoid. The spicules that are present are always surrounded by fluorescent marked cells. Bright field (C x200) shows embryoid derived from 16 mesomeres and 4 macromeres. Multiple gut-like structures appear in the central cavity of the epithelial sphere. Fluorescent field (D x200) shows that only one gut of all the gut-like structures present is surrounded by fluorescent cells. s=spicule g=gut.

Differentiation in meso: micro combinations (after 2 days)

<table>
<thead>
<tr>
<th>Ratio mesomeres:micromeres</th>
<th>% Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:0</td>
<td>80%</td>
</tr>
<tr>
<td>8:4</td>
<td>60%</td>
</tr>
<tr>
<td>16:0</td>
<td>40%</td>
</tr>
<tr>
<td>16:4</td>
<td>20%</td>
</tr>
</tbody>
</table>

Fig. 9. Differentiation of mesomeres cultured with macromeres. Differentiation obtained with mesomeres cultured alone, or with lineage tracer marked macromeres, at two different ratios. Most of the embryoids formed authentic gut-like structures. About 20% formed pigment cells derived from macromeres, and only 10% formed spicules. Survival of the cultures was 60% - 70%.
Discussion

Developmental potential of the mesomeres

The strategy for obtaining large numbers of different kinds of blastomeres from the 16-cell-stage embryo utilizes disaggregation into blastomere pairs in CFSW. Even though this is a mild regimen, and CFSW was used to 'loosen' blastomeres in classical experiments, the cell surface (and/or interior) may well have sustained alterations. However, a careful search for the effects of CFSW on mesomere differentiation did not reveal any effects of CFSW (Henry et al. 1989). While isolated mesomere pairs behave more or less according to classical expectations, there is a clear effect of reaggregating two or more mesomere pairs. They survive better in long-term culture and, after prolonged periods, long after normal differentiation and establishment of pattern has occurred in intact embryos, they display a clear ability to form many tissues of the embryo normally derived from different lineages of the vegetal hemisphere.

Recently Henry et al. (1989) have postulated that interactions among mesomeres may restrict their developmental potential during embryogenesis. This is based on two kinds of evidence: first, mesomeres obtained from different stages of the embryo, viz., 8-, 16- and 32-cell, showed a progressive decrease in ability to form vegetal structures within 72 h; and second, reaggregates of mesomeres and animal hemispheres from the 16-cell-stage embryo differentiate vegetal structures less extensively than do mesomeres pairs during the 72 h of culture. They observed that about 1/4 of both isolated mesomere pairs and aggregates of 8 mesomeres showed differentiation of vegetal characteristics within 3 days; this extent of differentiation is much greater than that observed here during the first 3 days in culture, but Henry et al. used mesomeres from embryos that displayed subequatorial, equatorial and supraequatorial 3rd cleavages, an important variable discussed before. When their experiments employed only equatorially cleaving zygotes, which are the only type used in all our experiments, only 9% of the embryoids showed gut formation, close to the 8% we observed. Similary low numbers were seen by Livingston and Wilt (1989, 1990). There is little difference in actual data when the same kinds of isolates are compared. Their conclusion that reaggregation restricts developmental potential of mesomeres is based on their expectation that if one pair of mesomeres shows vegetal characteristics 24% of the time, then aggregates of more than one pair of mesomeres should show much higher frequencies, an expectation that depends on the exact mechanisms by which vegetal characters develop. The fundamental difference in observations is that our conclusions of the effect of aggregation is based on prolonged survival and differentiation well past the 3 days, a time after the experiments of Henry et al. (1989) were terminated. Hence, insofar as direct comparison of results is possible, they are consistent. Our interpretation that aggregate size influences survival and differentiation is based on results obtained with cultures much older than those used in the study by Henry et al. (1989).

This delayed and atypical differentiation of mesomeres is certainly consistent with the plasticity of these cells in response to Li⁺, and clearly shows that there is extensive latent capacity of these cells to differentiate gut, spicules and pigment cells. It is also clear that there are homotypic interactions among these blastomeres that favor survival and enhance this latent capacity in long-term culture.

There are apparently interactions between mesomeres that may restrict the differentiation of vegetal characters during the first 3 days of development in culture; there are also interactions between blastomeres that favor extensive vegetal differentiation in long-term culture (more than 3 days), as shown here.

We may ask what is the meaning of this plasticity of mesomeres for understanding normal development. First, there is the possibility that during normal development there are mechanisms that suppress the latent ability of mesomeres to form such a large variety of tissues (Henry et al. 1989). Second, as the hoped for progress in understanding the mechanisms of cellular interactions in embryos occurs, models will have to reckon with the latent ability of mesomeres to form almost all the tissues of the larva. We propose that these latent abilities of mesomeres are governed by a delicately balanced regulatory network(s), and that over prolonged periods of time in culture the regulation directing normal mesomere fate breaks down.

Interactions of mesomeres

The results obtained with combinations of micromeres or macromeres with cells of the animal hemisphere seem at first glance what one could expect. In the combined cultures, there is a much wider range of differentiation than would occur in mesomeres alone under those conditions of culture and, hence, cell interactions must have occurred.

Micromeres evoke gut differentiation in mesomeres, differentiate themselves into spicules and stimulate a very low level of pigment cell appearance. Varying the number of micromeres with a given number of mesomeres does not change this basic response.

Macromeres also self-differentiate to form gut spicules and pigment cells when they are combined with mesomeres, and all these tissues would be expected to form from macromeres. Varying the number of mesomeres with a given number of macromeres does not change this basic response, but when a large number of mesomeres are present, there may be instances of multiple gut formation. The lineage tracing also indicates that mesomeres have been induced by macromeres to participate in gut formation in addition to the contribution of macromeres themselves to gut development. We wish to underline that the quantitation of the inductive response used here is all or none; there either is a gut, a spicule, a pigment cell, or there isn't. Since the inducing micromeres or macromeres are themselves able to form spicules, it is essentially the extent of gut formation from mesomeres that could be variable in
individual embryoids, and though we were not struck by this, it is hoped to make quantitative measurements of this type in future experiments.

There are three interesting considerations that arise from these results. First, what prevents mesomeres from participating in gut formation in normal development. One possibility is that the veg 1 tier derived from macromeres does not have the ability to induce gut in mesomeres and that it is only the veg 2 tier, which never is normally contiguous with mesomeres, that is effective in the reaggregates. This idea can be tested. Another possibility is that the morphogenetic potentialities of macromeres, and perhaps micromeres to some extent, are so powerful that they organize invaginations and archenteron formation from whatever adjacent tissue is at hand, and that the surface epithelium is prevented from doing this in the intact embryo largely by mechanical constraints of morphogenesis.

A second question is why we failed to observed some quantitative effect of varying the micromere/mesomere ratio, the effect observed by Horstadius that has been enshrined in textbooks for decades. It may simply be that the dissociation technique used here obliterates this subtlety. We think that it is more likely, however, that forming recombinant cultures by transplantation allows presence of a single center (one or several micromeres, or macromeres) that can initiate morphogenesis and gastrulation, and that the primary effects seen by Horstadius were a consequence of more and more normal pattern formation due primarily to morphogenetic consequences of the presence of vegetal cells, rather than some simple effect of whether a particular kind of differentiation was evoked. In other words, the effect of vegetal cells on animal cells may have as much to do with organization of proper morphogenetic movements as it does with kinds of differentiation induced. Observation of morphogenetic movements in such recombinants may help evaluate this hypothesis.

Third, we may ask about the morphogenetic behavior of primary and secondary mesenchyme in these recombinant cultures. Ettensohm and McClay (1986) have clearly shown that mesenchyme undergoes a stereotyped set of morphogenetic movements culminating in formation of mesenchymal clusters that form spicules. In cases where several micromeres are incorporated into a reconstituted embryo one might expect, then, that their descendants would ingress into the embryoid into a reconstituted embryoid one might expect, then, in situ hybridization using asymmetric RNA probes. Devel Biol. 101, 485–502.

We would like to thank Robin Shaw for technical assistance, and laboratory colleagues Brian Livingston and Chris Killian for their comments and assistance.

References


(Accepted 20 March 1990)