Development 119, 833-840 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993



# Cell-cell interactions regulate skeleton formation in the sea urchin embryo

**Norris Armstrong[1], Jeff Hardin[2] and David R. McClay[1]**

[1]Department of Zoology, Duke University, Durham, NC 27708, USA
[2]Department of Zoology, University of Wisconsin, 1117 W. Johnson Street, Madison, WI 53706, USA

## SUMMARY

**In the sea urchin embryo, the primary mesenchyme cells (PMCs) make extensive contact with the ectoderm of the blastula wall. This contact is shown to influence production of the larval skeleton by the PMCs. A previous observation showed that treatment of embryos with $NiCl_2$ can alter spicule number and skeletal pattern (Hardin et al. (1992) *Development,* 116, 671-685). Here, to explore the tissue sensitivity to $NiCl_2$, experiments recombined normal or $NiCl_2$-treated PMCs with either normal or $NiCl_2$-treated PMC-less host embryos. We find that $NiCl_2$ alters skeleton production by influencing the ectoderm of the blastula wall with which the PMCs interact. The ectoderm is responsible for specifying the number of spicules made by the PMCs. In addition, experiments examining skeleton production in vitro and in half- and quarter-sized embryos shows that cell interactions also influence skeleton size. PMCs grown in vitro away from interactions with the rest of the embryo, can produce larger spicules than in vivo. Thus, the epithelium of the blastula wall appears to provide spatial and scalar information that regulates skeleton production by the PMCs.**

Key words: sea urchin, skeleton, pattern, mesenchyme, $NiCl_2$

## INTRODUCTION

Epithelial-mesenchymal interactions play an important role during the development of many organisms. These interactions are associated with changes in cell fate, cell behavior and, in some instances, are required for the formation of specific organs and structures. In the sea urchin embryo, primary mesenchyme cells (PMCs) make extensive contact with the ectodermal epithelium of the blastula wall. In this study, we examine, experimentally, the role of interactions between the PMCs and the presumptive ectoderm during skeleton production.

The formation of the larval skeleton is easy to observe in the transparent sea urchin embryo and the sequence of events involved in this process has been well described (for review see: Okazaki, 1975a; Solursh, 1986; Wilt, 1987). After ingressing into the blastocoel, the PMCs migrate along the wall of the blastocoel then aggregate to form a ring of cells around the base of the invaginating archenteron. Larger numbers of PMCs accumulate at two sites along the ring and are referred to as the ventrolateral clusters. Skeleton production is initiated within these two clusters with the formation of two triradiate spicules. These spicules grow to form separate halves of the completed skeleton in a pattern that is species specific.

Several lines of evidence indicate that PMC behavior and skeleton production are influenced by the embryonic environment. For example, the timing of mesenchymal ring formation and skeleton production by the PMCs are responsive to cues external to the PMCs. If migratory (older) PMCs are transplanted into the blastocoels of younger embryos, the transplanted cells become inactive and remain this way until the host embryo reaches the mesenchyme blastula stage. The older donor cells then resume migration and produce a normal skeleton in concert with the host PMCs (Ettensohn and McClay, 1986). The embryonic environment also appears to limit the size of the skeleton produced by the PMCs. Previous experiments have shown that half-, quarter- and giant-sized embryos produce proportionally sized skeletons (Driesch, 1900; Hörstadius, 1957) indicating that skeleton size is somehow coordinately regulated with embryo size. This regulation appears to be imposed on the PMCs by the rest of the embryo as large numbers of supernumerary PMCs can be added to an embryo without affecting the size of the skeleton produced (Ettensohn, 1990). Finally, formation of the correct skeletal pattern requires interaction of the PMCs with the rest of the embryo. Both micromeres and PMCs can be isolated and grown in culture where, under the appropriate conditions, they will produce spicules (Ettensohn, 1990; Harkey and Whiteley, 1980; Okazaki, 1975b). In the absence of interactions with the rest of the embryo, however, the spicules formed in these cultures fail to attain the complex morphology seen in vivo (Kinoshita and Okazaki, 1984).

In this study, we begin an examination into how interactions between the PMCs and the rest of the embryo influences skeleton production. To do this, we have taken advantage of the observation that treatment of embryos with $NiCl_2$ alters skeleton production (Hardin et al., 1992). By transplanting normal and $NiCl_2$-treated PMCs into normal and $NiCl_2$-treated PMC-less host embryos, we demonstrate that interaction between the PMCs and the epithelium of the

blastula wall influences several aspects of skeleton production including the number of the spicules produced by the PMCs as well as the pattern of completed skeleton. Furthermore, experiments examining skeleton production in vivo and in vitro indicate that cell interactions also influence skeleton size.

## METHODS AND MATERIALS

### Animals and embryos

*Lytechinus variegatus* adults were collected at the Duke University Marine Laboratory (Beaufort NC) or were obtained from a commercial supplier (Susan Decker Services, Hollywood, FL). *Strongylocentrotus purpuratus* adults were obtained from Marinus Inc. (Long Beach, CA). Gametes were collected by inducing adults to spawn with intracoelomic injection of 0.5 M KCl. After collection, the eggs were washed in artificial sea water (ASW) (after Hinegardner, 1967) and were fertilized with a dilute suspension of sperm. Dilute concentrations of embryos were grown in ASW.

### Chemical treatment of embryos

Treatment of embryos with $NiCl_2$ was carried out essentially as described by Hardin et al. (1992). Briefly, embryos were grown in a solution of 1 mM $NiCl_2$ from fertilization until the early gastrula stage. To remove the $NiCl_2$, embryos were pelleted and resuspended a minimum of three times in ASW. Inflation of the blastocoel with sucrose treatment was modified after Moore and Burt (1939). Embryos were grown from fertilization onward in a solution of 0.76 M sucrose in $dH_2O$ mixed 3:7 with ASW. At the mesenchyme blastula stage or later, the embryos were washed free of sucrose by pelleting and resuspending 2× in ASW.

### Manipulation of embryos

Removal and transplantation of PMCs was carried out as previously described by Ettensohn and McClay (1986). PMCs were removed from host embryos by immobilizing the embryos in a Kiehart microinjection chamber (Kiehart, 1982) and flushing the blastocoel with ASW expelled from a fine glass needle. For $NiCl_2$-treated embryos, the Kiehart chambers were filled with 1 mM $NiCl_2$ and the blastocoels were flushed with $NiCl_2$-containing sea water. After elimination of the PMCs, the manipulated embryos were removed from the chambers and cultured until the early gastrula stage in depression slides kept in humidified chambers. The host embryos were then placed into fresh manipulation chambers for transplantation. Prior to transplantation, both $NiCl_2$-treated donor and host embryos were rinsed a minimum of three times and incubated for at least 15 minutes in normal ASW to remove the $NiCl_2$. Transplanted PMCs were obtained from donor embryos that had been vitally labeled with 0.4 mg/liter solution of rhodamine isothiocyanate from fertilization onwards until transplantation (Ettensohn and McClay, 1986). In all instances, embryos containing fluorescently labeled cells were grown in darkened chambers. Transplantation of half-sized embryos was carried out in the same manner as full-sized embryos.

### Production of half- and quarter-sized embryos

Embryos were fertilized in 10 mM para-amino-benzoic acid and immediately rinsed through 73 μm nytex mesh to remove the fertilization membrane (McClay and Fink, 1982). The demembranated embryos were then washed 1× in calcium/magnesium-free ASW (CMF-ASW) and 3× in calcium-free ASW (CF-ASW). Embryos were cultured in CF-ASW under continuous agitation to dissociate the embryos into individual blastomeres. At the 2- and 4-cell stages, healthy individual blastomeres were collected by mouth pipet and transferred to 35×10 mm Petri dishes coated with 0.5% agar for culture.

### Micromere culture

Micromeres were isolated following a method modified after McClay and Fink (1982) and Harkey and Whiteley (1985). 16-cell-stage *Strongylocentrotus purpuratus* embryos were washed 3× in CF-ASW and 1× in CMF-ASW to dissociate the embryos into single blastomeres. The dissociated cells were then layered on top of a 2-8% linear sucrose gradient and allowed to settle for 30 minutes. The micromeres were collected as a thin band of cells near the top of the gradient and were concentrated by centrifugation at approximately 200 ***g*** for 5 minutes. The concentrated micromeres were then resuspended in ASW containing 2% horse serum and cultured in 24-well Costar tissue culture plates.

### Spicule measurements

Calcarious deposits in embryos were considered to be spicules if the deposits were in the form of triradiates (i.e. possessed three distinct arms). Calcarious granules not in the form of triradiates were not considered to be spicules and were not counted in experiments in which spicule number was determined.

Skeleton size was estimated as the total length of the individual spicule rods that made up the skeleton. Measurements of spicule length were made from photographs on a Summagraphic digitizing pad using MacMorph (Hardin, 1989) or SigmaScan (Jandel Scientific, Corte Madera, CA) morphometrics software.

### Statistical analysis

To determine if the experimental treatments significantly altered skeleton production, pairwize analyses of experimental and control groups were carried out using the Scheffe Multiple Comparison test using Statview II Statistical software (Calabasar, Ca). Groups that were determined to be different at greater than the 95% confidence interval ($P<0.05$) were considered to be significantly different.

## RESULTS

### $NiCl_2$ affects skeleton pattern by altering ectodermal development

In embryos treated with $NiCl_2$, the PMCs produce multiple spicules around the ventral perimeter of the blastocoel (Hardin et al., 1992). This contrasts with normal embryos, which produce just two spicules in the ventrolateral clusters. One way in which $NiCl_2$ might influence spicule formation is if $NiCl_2$ influences the PMCs directly promoting the production of supernumerary spicules. Alternatively, $NiCl_2$ may alter spicule formation by influencing the tissues with which the PMCs interact. For example, examination of ectodermal markers indicates that ventral ectoderm is greatly expanded in $NiCl_2$-treated embryos while dorsal ectoderm is nearly eliminated (Hardin et al., 1992). As spicule production is normally initiated by PMCs located next to ventrolateral ectoderm, a possible outcome of an increase in the amount of ventral ectoderm in $NiCl_2$-treated embryos, might be an increase in the number of sites where spicule formation can take place.

To determine whether $NiCl_2$ alters skeleton production by influencing the PMCs or the ectoderm, we transplanted $NiCl_2$-treated PMCs into untreated control embryos that had been depleted of their own PMCs. In the converse experiment, untreated control PMCs were transplanted into $NiCl_2$-treated PMC-less hosts. If $NiCl_2$ promotes formation of extra spicules by altering the ectoderm, then embryos with

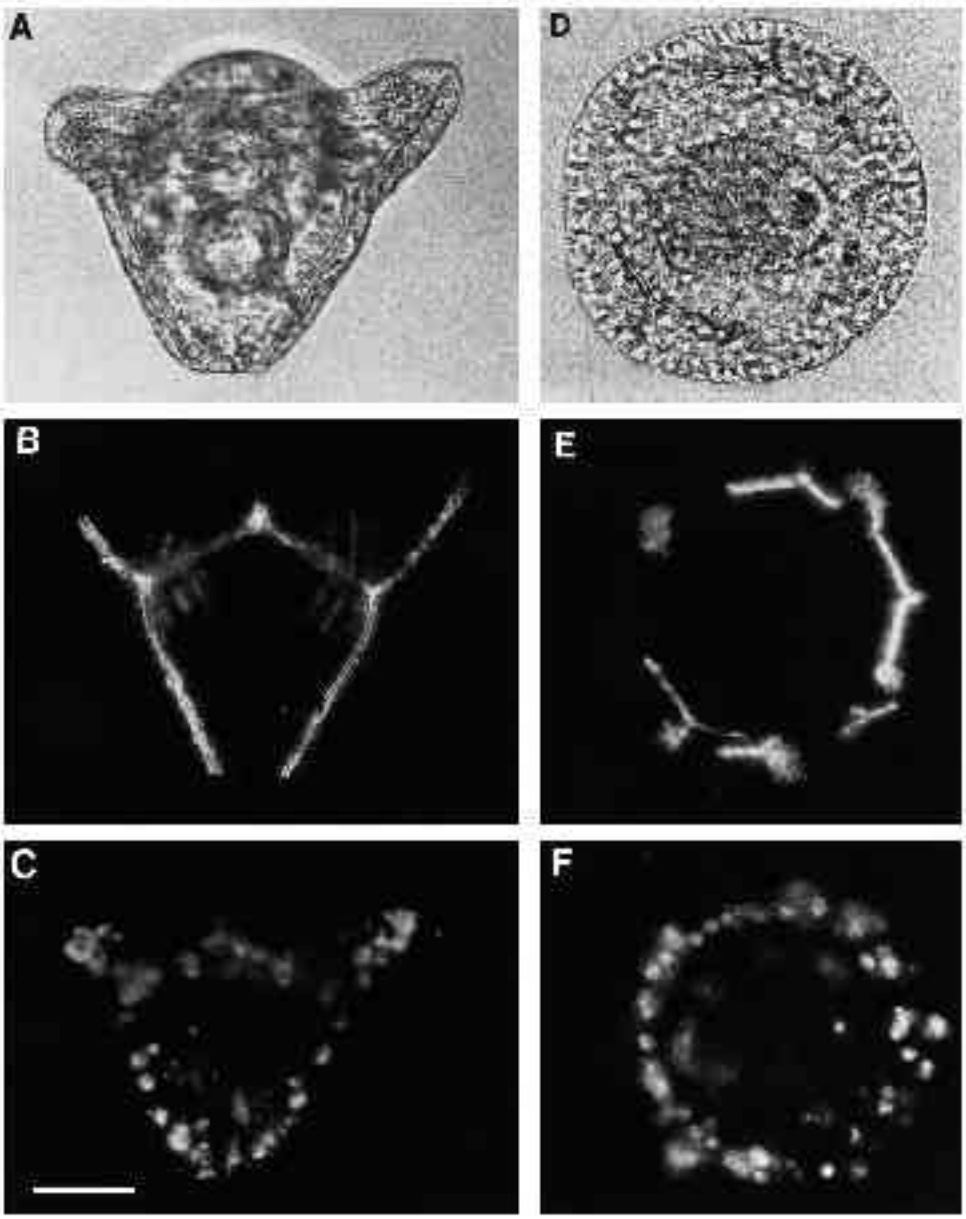


**Fig. 1.** Skeleton production in normal/$NiCl_2$-treated chimeric embryos. (A-C) $NiCl_2$-treated PMCs recombined with normal ectoderm. (D-F) Normal PMCs recombined with $NiCl_2$-treated ectoderm. (A,D) Bright field. (B,E) Birefringent spicules viewed under crossed polarizers. (A-C) Chimeric embryos containing normal ectoderm and $NiCl_2$-treated PMCs developed with bilateral symmetry and produced just two spicules with normal morphology. (D-F) Embryos containing normal PMCs and $NiCl_2$-treated ectoderm developed with radialized symmetry and produced multiple spicules that developed as enlarged triradiates and lacked the normal branching pattern. Examination of embryos under epifluorescence (C,F) revealed that most transplanted cells were closely associated with the $CaCO_3$ spicules. The embryos in D-F have been compressed under a glass coverslip to enable accurate counts of spicule number. Scale bar equals 50 μm.

untreated ectoderm should always produce normal skeletons while embryos with $NiCl_2$-treated ectoderm should always produce extra spicules. If $NiCl_2$ influences the PMCs directly, however, then the pattern of the skeleton produced in the chimeras would depend on the source of the PMCs. To ensure that untreated tissues were not exposed to $NiCl_2$, treated embryos were washed free of $NiCl_2$ prior to transplantation.

Chimeric embryos were allowed to develop for 24 hours and examined for skeletal pattern. All 13 embryos containing normal PMCs in $NiCl_2$-treated ectoderm produced multiple spicules in a radialized pattern that was indistinguishable from unmanipulated $NiCl_2$ controls (Fig. 1D,E). In contrast, all 9 embryos containing normal ectoderm and $NiCl_2$-treated PMCs produced bilaterally symmetric skeletons that consisted of just two spicules (Fig. 1A,B). To determine the fate of the transplanted cells, the donor PMCs were vitally labeled with rhodamine prior to each experiment. Examination of the experimental embryos by epifluorescence revealed that the transplanted PMCs were present and closely associated with the spicules (Fig. 1C,F). Thus $NiCl_2$ appears to cause production of supernumerary spicules by affecting the ectoderm.

### $NiCl_2$-treated embryos continue to regulate skeleton formation

Previous experiments have shown that extra PMCs can be added to normal embryos without noticeably augmenting the number of skeletal elements. This indicates that the embryonic environment somehow regulates the number and size of the spicules that the PMCs produce (Ettensohn, 1990). Given those results, one way for $NiCl_2$ to permit production of supernumerary spicules would be if $NiCl_2$ eliminated regulation of the PMCs. This would allow the PMCs to produce more spicules than usual. To examine for this possibility, $NiCl_2$-treated embryos were transplanted with

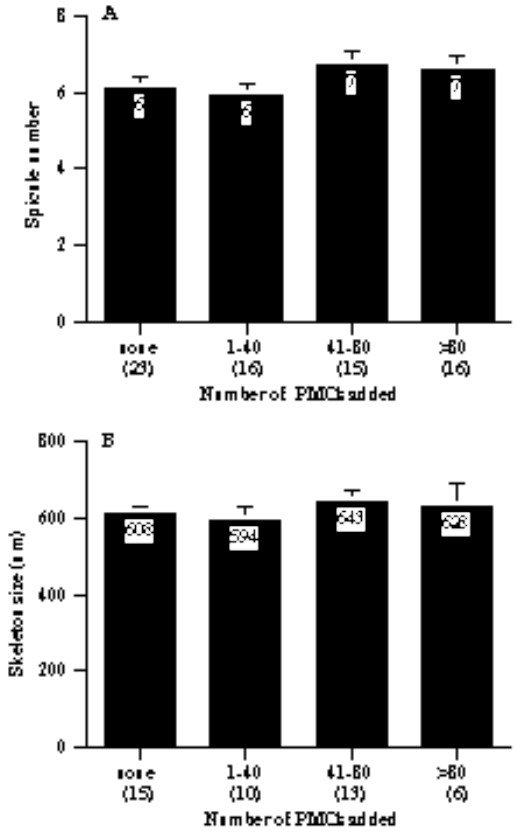


**Fig. 2.** (A,B) Spicule number and skeleton mass vs. PMC number $NiCl_2$-treated embryos. $NiCl_2$-treated embryos were transplanted with up to 130 supernumerary PMCs to determine the ability of these embryos to regulate skeleton production. Transplanted embryos were examined for spicule number (A) and skeleton size (B) 24 hours after manipulation. Skeleton size was estimated as the total linear length of the spicule rods that made up the skeleton. The number of spicules and the sizes of the skeletons produced in PMC-supplemented embryos were not significantly different from unmanipulated $NiCl_2$-treated controls (Scheffe Multiple Comparison test; $P<0.05$) indicating that spicule number and skeleton size are still regulated in $NiCl_2$-treated embryos. Mean ± s.e.m. is indicated in each case.

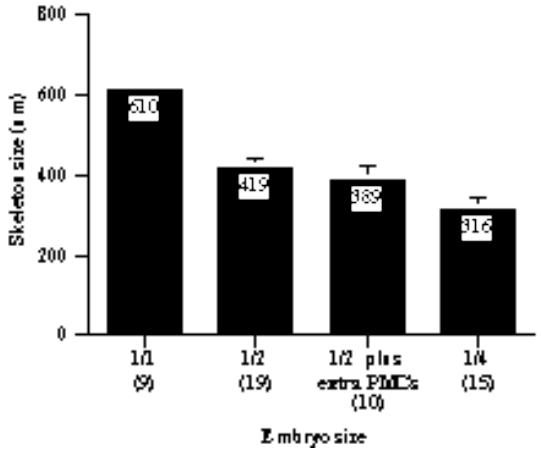


**Fig. 3.** Skeleton size vs embryo size. The possibility that reduced skeleton size in smaller embryos is due to the presence of fewer PMCs was tested by comparing skeleton production in quarter-, half- and full-sized embryos with half-sized embryos transplanted with supernumerary PMCs. Skeleton size decreased significantly with decreases in embryo size (Scheffe Multiple Comparison test; $P<0.05$). However, skeleton size was not significantly different between half-sized embryos transplanted with extra PMCs and half-sized controls ($P>0.05$) indicating that differences in skeletal size are not due to differences in PMC number. Skeleton size was estimated as the total length of the spicule rods making up the skeleton. Mean ± s.e.m. is indicated in each case.

up to two times the normal complement of PMCs. If treatment with $NiCl_2$ had eliminated regulation of spicule number, then addition of extra PMCs into the blastocoel should further increase the number of extra spicules produced in these embryos. If spicule number were still regulated, however, then no change in spicule number should occur. Alternatively, extra PMCs might result in the production of larger but not more numerous spicules. To test for this possibility, we also determined the total length of the skeleton produced in treated PMC-augmented embryos.

It was found that neither spicule number nor skeleton size increased significantly with the addition of extra PMCs to the blastocoel relative to $NiCl_2$-treated control embryos (Fig. 2A,B). The lack of larger or more numerous spicules in transplanted embryos was not due to the failure of the transplanted PMCs to take part in spicule formation. Examination of transplanted embryos under epifluorescence showed that virtually all of the transplanted cells were closely associated with the spicules. Thus, even though more spicules are produced in $NiCl_2$-treated embryos relative to controls, the embryo still appears to limit spicule number and size.

## Skeleton size is responsive to embryonic scale

Previous studies have shown that blastomeres isolated from 2- and 4-cell-stage embryos can develop into normal half- and quarter-sized embryos respectively (Driesch, 1900). These embryos, however, also have approximately half and quarter the normal complement of PMCs (Takahashi and Okazaki, 1979). To determine if half- and quarter-sized embryos produced smaller skeletons because they had fewer PMCs, we added extra PMCs to half-sized embryos and measured the skeletons that they produced. It was found that half-sized embryos containing extra PMCs produced skeletons that were approximately the same size as half-sized controls (Fig. 3). Therefore, skeleton size in half- and quarter-sized embryos is not limited by the number of PMCs in the embryo but rather is limited by constraints imposed by environment surrounding the PMCs. This raises the obvious question then, if the PMCs are freed from embryonic constraints would they be able to produce spicules larger than in vivo? To determine this, we isolated micromeres and grew them in culture away from interaction with the rest of the embryo. When spicules produced in vivo and in vitro were compared after 5 days of culture, it was found that, although spicule size was highly variable in culture, in many instances, the isolated PMCs were able to produce spicules that were longer than the spicules produced in vivo (Table 1). Furthermore, when the spicules were examined on a length/cell basis, PMCs grown in culture were found to contribute twice as much to spicule length than PMCs in vivo. Thus, these results indicate that, when

**Table 1. Spicule formation in vivo and in vitro**

| | (N) | Spicule length | Cells/spicule | Spic. length/cell |
|---|---|---|---|---|
| In vivo spicules | 20 | 20.24±2.31 | 16* | 1.27±0.14 |
| In vitro spicules | 40 | 21.77±7.81 | 9.5±4.8 | 2.5±0.70 |

*Based on the assumption that *S. purpuratus* embryos have a minimum of 32 PMCs and that, on average, half of these cells will take part in the production of one of the embryo's two spicules.

freed from embryonic constraints, PMCs can produce longer than normal spicules.

### Spicule number in $NiCl_2$-treated embryos is responsive to embryo size

When grown in $NiCl_2$, normal-sized embryos produce an average of 6 spicule primordia. Would scale affect the production of spicules in $NiCl_2$-treated embryos? To decrease size, embryos were grown from blastomeres isolated from 2- and 4-cell-stage embryos. To increase size, embryos were grown in a sucrose/ASW solution until the mesenchyme blastula stage. When washed into normal ASW, osmotic pressure (exerted by the sucrose trapped in the blastocoel) caused these embryos to swell dramatically, greatly increasing the volume of the blastocoel (Moore and Burt, 1939).

When the skeletons produced by $NiCl_2$-treated embryos were examined, it was found that spicule number varied directly with differences in the size of the embryos (Fig. 4). The larger the embryo, the greater the number of spicules produced. Half- and quarter-sized embryos produced fewer spicules than full-sized $NiCl_2$-treated controls (Fig. 5), while swollen embryos had an even greater number of spicules (Fig. 6). A reduction in spicule number in smaller embryos was not due to the decreased numbers of PMCs in these embryos. Half-sized embryos that had been transplanted with extra PMCs produced the same number of spicules as half-sized $NiCl_2$-treated controls (Fig. 5). Strikingly, embryos that had not been treated with $NiCl_2$ always produced just two spicules regardless of the size of the embryo (data not shown). This suggests that spicule number is regulated in $NiCl_2$-treated embryos by some mechanism that is not influenced by embryo size during normal development.

To determine when embryo size matters for specification of spicule number, we grew $NiCl_2$-treated embryos in sucrose and washed them into normal sea water at various stages of development. Continuous culture of embryos in sucrose has no significant influence on embryo morphology although blastocoel size can be somewhat reduced. It was found that the maximum number of spicules were produced if the embryos were swollen prior to the early gastrula stage (Fig. 6). This corresponds to the time when skeleton formation is first initiated. If embryos were swollen at increasingly later stages, the embryos eventually made as many or fewer spicules as in unswollen $NiCl_2$-treated control embryos. Thus there appears to be a timing and a territorial character to regulation of spicule production.

## DISCUSSION

This study demonstrates that several aspects of skeleton production, including spicule number, spicule size and at least

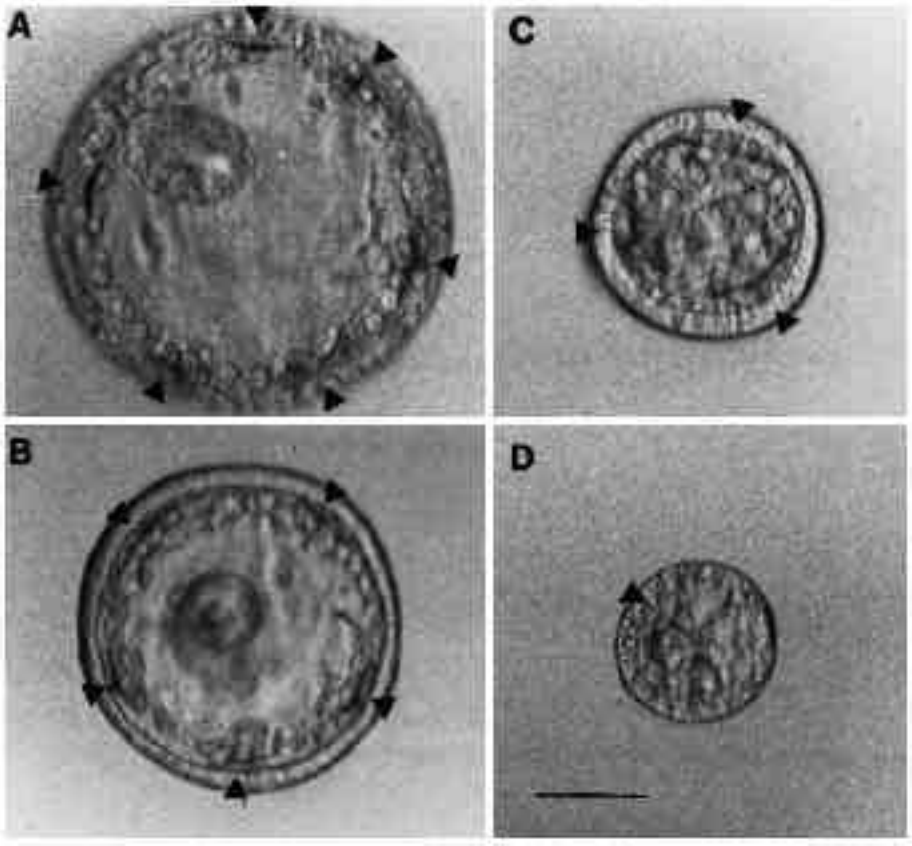


**Fig. 4.** Spicule number vs. embryo size in $NiCl_2$-treated embryos. To test for possible mechanisms by which skeleton production might be regulated in $NiCl_2$-treated embryos, spicule number was examined in embryos whose sizes had been experimentally altered. (A) $NiCl_2$-treated embryo grown in 0.76 M sucrose solution mixed 1:3 with ASW until the mesenchyme blastula stage. (B) control $NiCl_2$-treated embryo. (C) Half-sized $NiCl_2$-treated embryo. (D). Quarter-sized $NiCl_2$-treated embryo. Arrowheads mark where spicules have been produced. Scale bar equals 50 μm.

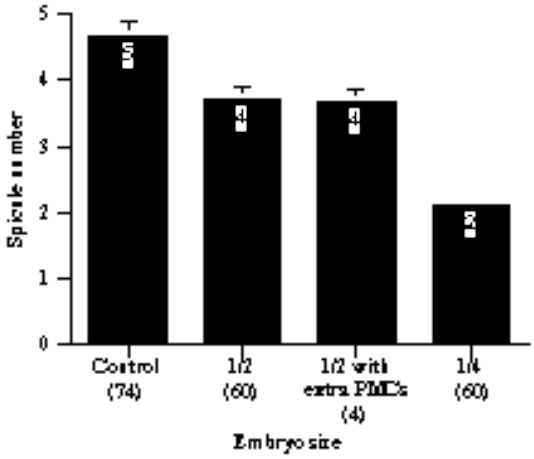


**Fig. 5.** Analysis of spicule production in different sized $NiCl_2$-treated embryos. To determine if the number of spicules produced in different sized embryos were statistically significant, spicule production in full-, half- and quarter-sized embryos were analysed using the Scheffe Multiple Comparison test. Spicule number was found to decrease significantly with decreases in embryo size ($P$<0.05). However, spicule number was not significantly different between half-sized $NiCl_2$-treated embryos transplanted with extra PMCs and half-sized $NiCl_2$-treated controls ($P$>0.05) indicating that changes in spicule number were not due to differences in PMC number. Mean ± s.e.m. is indicated in each case.

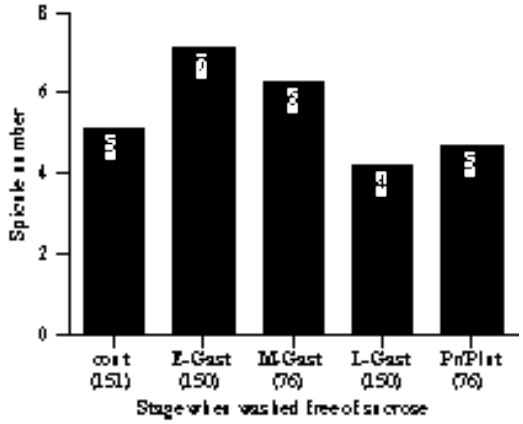


**Fig. 6.** Spicule number vs. stage at which blastocoel size is increased. The timing of the sensitivity of spicule number to increases in embryo size was tested by swelling embryos at varying time points after PMC ingression as described in Methods and Materials. Significantly more spicules were produced by embryos washed out of sucrose at the early and mid-gastrula stages relative to $NiCl_2$-treated control embryos (Scheffe Multiple Comparison test; $P$<0.05). Significantly fewer spicules were produced in embryos washed out of sucrose at the late gastrula stage relative to $NiCl_2$-treated controls ($P$<0.05). Spicule number in embryos washed out of sucrose at the prism/pluteus stage was not significantly different from controls ($P$>0.05). Mean ± s.e.m. is indicated in each case.

some aspects of skeletal pattern are influenced by interactions of the PMCs with the rest of the embryo. These conclusions are based on a number of experimental observations. First, the ability of $NiCl_2$ to alter skeletal pattern is due, not to the influence of $NiCl_2$ on the PMCs producing the skeleton, but rather to the influence of $NiCl_2$ on the ectoderm with which the PMCs are in contact. PMCs taken from embryos treated with $NiCl_2$ are still able to produce normal skeletons if transplanted into normal PMC-less host embryos. Normal PMCs recombined with $NiCl_2$-treated PMC-less hosts, however, always produce supernumerary spicules with abnormal morphology. Second, skeleton size in vivo appears to be limited by the size of the embryo and is independent of the number of PMCs in the blastocoel. PMCs isolated in culture, however, appear to be free of embryonic constraints and can produce longer spicules than in vivo. Combined with previous experiments (Ettensohn and McClay, 1986), our results indicate that the ectoderm contributes to spatial, temporal and scalar control of skeletogenesis through (presumably) close range interactions with primary mesenchyme cells.

## The ectoderm interacts with PMCs to influence skeleton production

During migration and skeleton production, the PMCs make extensive contact with the ectoderm of the blastula wall and it has been suggested that the ectoderm is responsible for regulating PMC behavior and skeleton production (Wolpert and Gustafson, 1961; Harkey and Whiteley, 1980; Ettensohn, 1990). In support of this possibility, isolated PMCs are able to produce spicules in vitro but, unless the PMCs are allowed to reaggregate with ectoderm, these spicules fail to develop with the correct morphology (Harkey and Whiteley, 1980). Comparison of skeleton production in normal and $NiCl_2$-treated embryos also supports the idea that the ectoderm influences skeleton production. In normal embryos, skeleton production is initiated by PMCs located next to regions of ventral (oral) ectoderm that are morphologically distinct from ectoderm in the rest of the embryo. The ectoderm in these regions is thicker than in other regions and the cells are arranged in an overlapping pattern (Okazaki, 1960; Galileo and Morrill, 1985). In $NiCl_2$-treated embryos, these regions of ectoderm appear to be expanded; instead of forming two distinct regions laterally in the embryo, the thickened ectoderm develops as a band that encircles the vegetal pole. In these embryos, the PMCs do not form distinct ventrolateral clusters and produce spicules at multiple sites (Hardin et al., 1992).

These observations suggest that the PMCs produce the skeleton in response to cues from the ectoderm. It is possible that the thickened regions of ventral (oral) ectoderm promote spicule formation or, alternatively, spicule formation may be inhibited by dorsal (aboral) ectoderm. In either instance, expansion of ventral (oral) ectoderm at the expense of dorsal (aboral) ectoderm in $NiCl_2$-treated embryos should enable PMCs to produce extra spicules. We cannot rule out the possibility, however, that other tissues may also influence skeleton production. For example, as they aggregate to form the mesenchymal ring, the PMCs also make contact with the presumptive endoderm of the vegetal plate. Cell lineage analysis has shown that, as with the ectoderm, the vegetal plate is also polarized with respect to the dorsoventral (oral-aboral) axis (Cameron et al., 1991). It is formally possible that polarization of the vegetal plate endoderm is affected by treatment of embryos with $NiCl_2$ and that such changes in the vegetal plate may in turn

influence skeleton production. The regions of influence could also be defined by the circular boundary between the ectoderm and the vegetal plate.

## How is skeleton production influenced by cell interactions?

One model for how cell interactions influence skeleton production in sea urchins proposes that the PMCs accumulate in regions of the embryo where they adhere most strongly. In this model, a prepattern of adhesion molecules in the embryo serves as a template for skeleton production by the PMCs (Wolpert and Gustafson, 1961). In support of this possibility, differences in cell adhesion have been proposed to influence cell migration and pattern formation in other systems (Dodd and Jessel, 1988; Hynes and Lander, 1992) and several molecules known to influence cell behavior and migration in other systems have been found in the basal lamina over which the PMCs migrate (see Solursh for review, 1986). Furthermore, the PMCs do appear to adhere more strongly to the blastula wall after they have aggregated to form the mesenchymal ring (i.e. they are more difficult to remove from the embryo during transplantation experiments, Ettensohn, 1990). It is not known, however, whether the increase in adhesion of PMCs is due to qualitative differences in regions of the blastula wall or to changes in the behavior of the PMCs in response to some other signal. Also, although the basal lamina has been shown to promote directed migration of PMCs in vitro, this ability appears to be independent of adhesion strength (Solursh and Lane, 1988).

Another way in which cell interactions could influence skeleton production is if the embryo provides positional information that the PMCs interpret. In this model, regional differences in the concentration, presence or absence of certain molecules presented by the embryo would inform the PMCs as to their location. Then, on the basis of the information that they receive, each PMC would behave according to its location. In support of this model, we show elsewhere (Armstrong and McClay, unpublished data) that chimeric embryos made from PMCs and ectoderm from different species of sea urchin produce skeletons in the pattern of the PMC donor. This observation argues against the presence of a prepattern in the ectoderm and suggests instead that skeletal pattern is determined autonomously by the PMCs. The ability of the PMCs to produce the skeleton in the correct orientation in the host embryo, however, indicates that the PMCs respond to positional cues provided by the ectoderm. Those cues can be positive or negative. We generally think in terms of positive signals but it is formally possible that spicule primordia arise from the only two regions that are not inhibitory for spicule production.

## Skeleton production may be regulated by multiple mechanisms

Although $NiCl_2$-treated embryos produce supernumerary spicules, in this paper, we show that the number of spicules that can be produced in these embryos is still under some kind of regulation. How can production of an increased but limited number of spicules be accounted for? One possibility is that treatment with $NiCl_2$ increases spicule number simply by duplicating the ventral half of the embryo. As a result, twice as many spicules (four) would be expected to be produced as in untreated controls. This model does not appear to be correct, however, as normal-sized $NiCl_2$-treated embryos typically produce six spicules. An alternative possibility is that the spacing of the spicules produced in the embryo is somehow regulated. This possibility is suggested by the observation that the spicules produced in $NiCl_2$-treated embryos tend to be rather evenly distributed around the mesenchymal ring (unpublished observations). Furthermore, spicule number increases and decreases with increases or decreases in embryo size. Again, this would be expected if the number of spicules that could form within a given area were regulated. Regulation of spicule spacing could easily be accomplished if each spicule-forming center prevented additional spicules from forming nearby. Previous observations indicate that the spicule-forming centers, in fact, do interact with surrounding cells and tissue. For example, if the regions of ectodermal thickening associated with ventrolateral cluster formation are destroyed, neighboring ectoderm will form new sites around which the PMCs will aggregate instead (Czihak, 1962). It is also possible that regulation occurs through interactions between the PMCs themselves. Calcareous granules can often be seen at multiple sites along the mesenchymal ring but only those located in the ventrolateral clusters continue to develop (Okazaki, 1960; Ettensohn, 1990). If PMCs are prevented from joining the syncytium extended from the ventrolateral clusters, however, calcarious granules that these PMCs produce can be enlarged to form spicules (Okazaki, 1960). Thus, cell interactions somehow provide a territorial spacing component to the sites of spicule origin.

Normal embryos always produce two spicules regardless of their size. This observation might be explained, however, if one considers one or possibly two levels of regulation. First, the ventrolateral area is specified by the ectoderm-vegetal plate. Second, ventrolateral clusters containing large numbers of PMCs produce just one spicule each as a result of close range inhibition of supernumerary spicule formation. Thus, whereas interaction between the PMCs and the rest of the embryo appears to limit the number of sites where spicule formation can take place, additional interactions may function to regulate the number of spicules formed at these sites.

In summary, although much remains to be learned, we have provided evidence that interactions between the PMCs and the ectoderm/base plate regulates several aspects of skeletogenesis including spicule number, spicule size and skeletal pattern. Closer examination of the development of the dorsoventral (oral-aboral) axis and of PMC-ectoderm interactions should provide further insight into how skeleton production influenced.

The authors would like to thank Seth Ruffins and Chuck Ettensohn for their help with micromanipulation and sucrose treatment of embryos and Elizabeth McCain and Benjamin R. Johnson for help with manuscript preparation. Morphometrics analysis was carried out in the Duke University Morphometrics Laboratory. N. A. was supported in part by a CoCos Training Grant in Morphology. J. H. is a Lucille P. Markey scholar in the Biomedical Sciences. This research was funded by NSF grant DCB92-06872 to J. H. and NIH grant HD14483 to D. R. M.

## REFERENCES


**Cameron, R. A., Fraser, S. E., Britten, R. J. and Davidson, E. H**. (1991). Macromere cell fates during sea urchin development. *Development* **113**, 1085-1091.

**Czihak, G.** (1962). Entwicklungsphysiologische Untersuchungen an Echiniden. Topochemie der Blastula und Gastrula, Entwicklung der Bilateral- und Radiärsymmetrie und der Coelomdivertikel. *Wilhelm Roux's Arch. EntwMech. Org.* **154,** 29-55.

**Dodd, J. and Jessel, T. J**. (1988). Axon guidance and the patterning of neuronal projections in vertebrates. *Science* **242**, 692-699.

**Driesch, H.** (1900). Die isolirten Blastomeren des Echinidenkeimes. *WilhelmRoux Arch. EntwMech. Org.* **10,** 361-410.

**Ettensohn, C. A.** (1990). The regulation of primary mesenchyme cell patterning. *Dev. Biol*. **150,** 261-271.

**Ettensohn, C. A. and McClay, D. R.** (1986). The regulation of primary mesenchyme cell migration in the sea urchin embryo: transplantations of cells and latex beads. *Dev. Biol*. **117,** 380-391.

**Galileo, D. S. and Morrill, J. B.** (1985). Patterns of cells and extracellular material of the sea urchin Lytechinus variegatus (Echinodermata; Echinoidea) embryo, from hatched blastula to late gastrula. *J. Morph.* **185,** 387-402.

**Hardin, J.** (1989). Local shifts in position and polaried motility drive cell rearrangement during sea urchin gastrulation. *Dev. Biol.* **136,** 430-445.

**Hardin, J., Coffman, J. A., Black, S. D. and McClay, D. R.** (1992). Commitment along the dorsoventral axis of the sea urchin embryo is altered in response to $NiCl_2$. *Development* **116,** 671-685.

**Harkey, M. A. and Whiteley, A. H.** (1980). Isolation, culture, and differentiation of echinoid primary mesenchyme cells. *Roux's Archiv. Dev. Biol.* **189,** 111-122.

**Harkey, M. A. and Whiteley, A. H.** (1985). Mass isolation and culture of sea urchin micromeres. *In Vitro Cell. Dev. Biol.* **21,** 108-113.

**Hinegardner, R.** (1967). Echinoderms. In *Methods in Developmental Biology* (ed. F. H. Wilt and N. K. Wessels), pp. 130-155. New York: Thomas Y. Crowell.

**Hörstadius, S.** (1957). On the regulation of bilateral symmetry in plutei with exhanged meridional halves and in giant plutei. *J. Embryol. Exp. Morph.* **5,** 60-73.

**Hynes, R. O. and Lander, A. D.** (1992). Contact and adhesive specificities in the asociations, migrations, and targeting of cells and axons. *Cell* **88**, 303-322.

**Kiehart, D. P.** (1982). Microinjection of echinoderm eggs: apparatus and procedures. *Meth. Cell Biol.* **25,** 13-31.

**Kinoshita, T. and Okazaki, K.** (1984). In vitro study on morphogenesis of sea urchin larval spicule: adhesiveness of cells. *Zool. Sci.* **1,** 433-443.

**McClay, D. R. and Fink, R. D.** (1982). The role of hyalin in early sea urchin development. *Dev. Biol.* **92,** 285-293.

**Moore, A. R. and Burt, A. S.** (1939). On the locus and nature of the forces causing gastrulation in the embryos of Dendraster excentricus. *J. Exp. Zool.* **82,** 159-171.

**Okazaki, K.** (1960). Skeleton formation of the sea urchin larvae. II. Organic matrix of the spicule. *Embryologica* **5,** 283-320.

**Okazaki, K.** (1975a). Normal development to metamorphosis. In *The Sea Urchin Embryo* (ed. G. Czihak), pp. 177-232. Berlin: Springer-Verlag.

**Okazaki, K.** (1975b). Spicule formation by isolated micromeres of the sea urchin embryo. *Amer. Zool.* **15,** 567-581.

**Solursh, M.** (1986). Migration of sea urchin primary mesenchyme cells. In *Developmental Biology: A Comprehensive Synthesis, Vol. 2. The Cellular Basis of Morphogenesis* (eds. L. Browder), pp. 391-431. New York: Plenum Press.

**Solursh, M. and Lane, M. C**. (1988). Extracellular matrix triggers a directed cell migratory response in sea urchin primary mesenchyme cells. *Dev. Bio.* **130**, 397-401.

**Takahashi, M. M. and Okazaki, K.** (1979). Total cell number and number of the primary mesenchyme cells in whole, half- and 1/4 larvae of Clypleaster japonicus. *Dev. Growth Differ.* **21,** 553-566.

**Wilt, F. H.** (1987). Determination and morphogenesis in the sea urchin embryo. *Development* **100,** 559-575.

**Wolpert, L. and Gustafson, T.** (1961). Studies on the cellular basis of morphogenesis of the sea urchin embryo. Development of the skeletal pattern. *Exp. Cell. Res.* **25,** 311-325.


(Accepted 15 August 1993)