

ABSTRACT

Supervisor: Dr. R.D. Burke

The monoclonal antibody Sp12 recognizes an epitope shared by a diverse group of developmentally regulated cell surface and extracellular matrix antigens which are expressed during *Strongylocentrotus purpuratus* development. Immunofluorescent localization reveals antigen in the cortical granules of eggs and in the hyaline layer from fertilization through to gastrula. Primary (skeletogenic) mesenchyme and two secondary mesenchyme derivatives (blastocoelar cells and pigment cells) express antigen after their release into the blastocoel and maintain it throughout the remainder of larval development. The antibody allows detailed descriptions of blastocoelar cells, a prominent yet poorly described fibroblast-like mesenchyme lineage. In adults antigen is localized to the organic matrix which invests the calcified stereom of the test and spines. Immunogold electron microscopy shows antigen in the cortical granules of eggs, and on cell surfaces, within membrane bound vesicles, and within the Golgi apparatus of mesenchyme cells. On western blots a confluent smear of antigen (M_r primarily $>180K$) is present in eggs, but is resolved into seven antigen bands (M_r from $35K$ to $>200K$) after fertilization. A prominent antigen at $140K$ is newly expressed coincident with mesenchyme immunoreactivity. Antigens at $140K$ and $120K$ fade as prisms develop into plutei, while an antigen at $105K$ appears in older larvae. In adult test six antigens are shared with larvae, and there are two novel antigens at $75K$ and $110K$. Immunoreactivity is eliminated by digestion of samples with endoglycosidase F, is reduced by periodate oxidation, but is unaffected by boiling. Calcium-magnesium-free-seawater or $1M$ glycine extract a subset of antigens from dissociating embryos, leaving a complementary subset of antigens associated with cell membranes. Membrane antigens are not extracted, at $4^\circ C$, with high or low ionic strength, organic solvents, or non-ionic detergents but are solubilized by ionic detergents.

Whole antibody has no effect on development of embryos cultured in it from fertilization on, nor is there a specific effect from injection of antibody into the blastocoel of developing embryos. The shape of dissociated cells cultured in Sp12 whole antibody is markedly constrained compared to that of controls, however, this difference is not seen in cells incubated in Sp12 Fab fragments. Sp12 appears to recognize a carbohydrate moiety shared by ten glycoproteins of widely variable M_r and cell membrane affinity which are differentially expressed on mesenchyme throughout *S. purpuratus* development. The antibody does not disrupt development *in vivo*, but does affect cell shape *in vitro*, probably by crosslinking cell surface antigens.

Examiners:

Dr. R.D. Burke, Supervisor (Biology Department)

Dr. J.N. Owens, Departmental Member (Biology Department)

Dr. M.J. Ashwood-Smith, Departmental Member (Biology Department)

Dr. R.W. Olafson, Outside Member (Department of Biochemistry)

Dr. T.W. Pearson, Outside Member (Department of Biochemistry)

Dr. B.P. Brandhorst, External Examiner

TABLE OF CONTENTS

ABSTRACT	ii
TABLE OF CONTENTS	iv
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	x
ACKNOWLEDGMENTS	xiii
DEDICATION	xiv
FRONTISPIECE	xv
CHAPTER 1 INTRODUCTION	
The sea urchin embryo	1
Cytodifferentiation	5
Morphogenesis	7
Summary	22
Experimental rationale	23
CHAPTER 2 MONOCLONAL ANTIBODY PRODUCTION	
INTRODUCTION	24
MATERIALS AND METHODS	25
Sea urchin embryonic and larval culture	25
Immunization	26
Fusion	27
Cloning and ascites production	28
Antibody Purification	29
Fab fragment production	30
RESULTS	31
Immunization, fusion, and cloning	31
Antibody production, purification, and Fab generation	33

Tissue specificity	34
DISCUSSION	38
CHAPTER 3 <i>IN SITU</i> ONTOGENY OF THE SP12 ANTIGENS	
INTRODUCTION	41
MATERIALS AND METHODS	42
Embryonic and larval culture	42
Ontogeny	43
Immunogold localization	43
Controls for immunoreactivity	44
RESULTS	44
Ontogeny of the Sp12 antigens <i>in situ</i>	44
Sp12 antigen localization by immunofluorescence	47
Ultrastructural localization	48
Interspecific crossreactivity	49
DISCUSSION	64
Cortical granule and hyaline immunoreactivity	64
Mesenchyme specificity of Sp12	67
Ultrastructural localization	68
Adult tissue immunoreactivity	69
Species specificity of Sp12	71
CHAPTER 4 BIOCHEMICAL ONTOGENY AND ANALYSIS	
INTRODUCTION	73
MATERIALS AND METHODS	74
Polyacrylamide gel electrophoresis	74
Western blots	75
Immunoprecipitations	76
Proteolysis and epitope determination	77

Effect of developmental inhibitors on epitope expression	79
Membrane and ECM isolation	80
Extraction of membrane bound antigen	81
Cellular affinity of the antigens	81
RESULTS	83
Ontogeny of the antigens by western blot analysis	83
Crude extracts	83
Immunoprecipitates	84
Reduced versus unreduced samples	85
Proteolysis	86
Epitope determination	86
Effect of gastrulation inhibitors on epitope expression	87
Membrane-associated and extracellular antigen separation	89
Membrane bound antigen extraction	90
Cellular affinity of the antigens	91
DISCUSSION	107
Changing pattern of Sp12 antigen expression	107
Multiple antigens	111
Antigen membrane affinity	114
Antigen cyto-specificity	118
CHAPTER 5 MESENCHYME DERIVATIVES IN THE SEA URCHIN EMBRYO	
INTRODUCTION	120
MATERIALS AND METHODS	121
Immunofluorescence	121
Nomarski DIC and time-lapse observations	121
Electron microscopy	122
RESULTS	123

Mesenchyme derivatives in the sea urchin embryo	123
Blastocoelar cells	124
Blastocoelar cell ontogeny	126
Blastocoelar cell fine structure and behaviour	128
DISCUSSION	145
Sea urchin mesenchyme	145
Blastocoelar cells	145
CHAPTER 6 FUNCTION OF THE SP12 ANTIGENS	
INTRODUCTION	150
MATERIALS AND METHODS	151
Whole embryo incubations	151
<i>In vitro</i> analysis	152
<i>In vivo</i> analysis	153
RESULTS	154
Whole embryo incubations	154
<i>In vitro</i> analysis	155
<i>In vivo</i> analysis	158
DISCUSSION	168
Embryos cultured in antibody-FSW	168
Sp12 effects on cells <i>in vitro</i>	169
Injected Sp12 - ambiguous effect	172
CHAPTER 7 CONCLUSIONS	
Summary of findings - key points in the Sp12 investigation	174
Selected points for future investigation	176
LITERATURE CITED	178

LIST OF FIGURES

Figure 1. Life cycle of <i>Strongylocentrotus purpuratus</i> .	3
Figure 2. Tissue specificity of Sp12 in 5d plutei.	36
Figure 3. <i>In situ</i> ontogeny of Sp12 antigens.	50
Figure 4. NRH labelling of sea urchin embryos and larvae.	54
Figure 5. Mesenchyme derivatives labelled by Sp12.	56
Figure 6. High magnification views of Sp12 labelled cells in the pluteus.	56
Figure 7. Adult tissue specificity.	58
Figure 8. Immunogold localization of Sp12 antigen.	60
Figure 9. Tissue specificity of Sp12 in other species of echinoids.	62
Figure 10. PAGE separated proteins from 11 developmental stages of <i>S. purpuratus</i> .	93
Figure 11. Western blot of samples from a developmental series, probed with Sp12.	93
Figure 12. Western blot of immunoprecipitated samples from a developmental series.	95
Figure 13. Patterns of antigens under reducing and nonreducing conditions.	97
Figure 14. Artifactual proteolysis does not produce the multiple bands.	97
Figure 15. Proteolysis and periodate sensitivity of the Sp12 epitope.	99
Figure 16. Endoglycosidase F treatment of the Sp12 antigens.	99
Figure 17. Boiling does not reduce Sp12 immunoreactivity.	99
Figure 18. Effect of developmental inhibitors on Sp12 epitope expression.	101
Figure 19. Sp12 labelling of live cells.	103
Figure 20. Separation of ECM and membrane associated antigens.	103
Figure 21. Dot blot analysis of antigen extraction from crude membrane preparations.	103

Figure 22. Antigens in cell fractions.	105
Figure 23. Composite of western blots from analogous developmental stages.	105
Figure 24. The fate of gastrula stage mesenchyme cells in the pluteus larva.	131
Figure 25. Nomarski interference contrast micrographs of blastocoelar cells in the 5 d pluteus.	133
Figure 26. Sp12 immunofluorescence micrographs of blastocoelar cells.	133
Figure 27. High magnification views of immunolabelled blastocoelar cells.	133
Figure 28. Artist's three dimensional representation of the blastocoelar network in a 96 hr early pluteus.	135
Figure 29. Time lapse video sequence of gastrulation.	137
Figure 30. Nomarski differential interference contrast micrographs of blastocoelar cell development.	137
Figure 31. Graph showing number of blastocoelar cells versus hours PF.	139
Figure 32. Scanning electron micrographs of the blastocoel.	141
Figure 33. Blastocoelar cell activity in the 96 hr early pluteus.	143
Figure 34. Blastocoelar cell activity in a 7 d pluteus, monitored with timelapse video microscopy.	143
Figure 35. Effect of incubating live embryos in Sp12.	160
Figure 36. Effect of Sp12 on cells <i>in vitro</i> .	162
Figure 37. Low concentrations of Sp12 have a subtle effect on cell morphology <i>in vitro</i> .	164
Figure 38. Increasing concentrations of Sp12 produce increased effects on cell morphology.	164
Figure 39. Dissociated gastrula stage cells are also perturbed by incubation in Sp12.	164
Figure 40. Microinjection of antibody into developing embryos does not produce a specific defect.	166

LIST OF ABBREVIATIONS

AB	activation buffer
ASW	artificial sea water
βAPN	beta aminopropionitrile
BL	basal lamina
BSA	bovine serum albumin
CMFSW	calcium magnesium free sea water
d	day
DIC	Nomarski differential interference contrast optics
DTT	dithiothreitol
DW	distilled water
E-64	trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglutaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
Fab	fragment antigen binding
Fc	fragment crystallizable
FCS	fetal calf serum
FITC	fluoresceine isothiocyanate
FSW	filtered sea water
g	force of gravity
HAT	hypoxanthine/aminopterin/thymidine
HT	hypoxanthine/thymidine
hr	hour
HRP	horseradish peroxidase

IPAB	immunoprecipitation assay buffer
K	relative molecular mass, x1000
M	molar
M_r	relative molecular mass
mM	millimolar
μM	micromolar
mg	milligram
μg	microgram
ml	millilitre
μl	microlitre
mm	millimeter
μm	micrometer
nm	nanometer
MBL	Marine Biological Laboratory, Wood's Hole
MFSW	0.22 μm millipore filtered sea water
NRH	non-relevant hybridoma - antibody directed against RuBisCo plant enzyme.
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid Schiff's reagent
PBS	phosphate buffered saline
PEG	polyethylene glycol
PF	post-fertilization
PMSF	phenylmethanesulphonyl fluoride
RAMIG	rabbit anti-mouse immunoglobulin G
RPMI	tissue culture medium
SDS	sodium dodecylsulphate
SFSW	sulphate free sea water

Sp12	the twelfth monoclonal antibody produced in this lab, and the major reagent used in this study
TLCK	N-p-tosyl-L-lysine chloromethyl ketone
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone

ACKNOWLEDGMENTS

I am grateful to my supervisor, Dr. R.D. Burke, for his many contributions to this project. In addition to equipment, materials, funds, and instruction, he provided intellectual and personal support throughout my research and writing.

My colleagues have also aided in the completion of this work. I especially thank Dr. Allan Gibson, Rob Myers, and Dr. Catherine Bouland for their interest, comments, and technical expertise. Many others, both in the lab and in the faculty, have offered encouragement, and stimulating conversation. To them I also offer my thanks. The scientist does not live by test-tube alone!

The Natural Science and Engineering Research Council, the Medical Research Council of Canada, and the University of Victoria provided the bulk of my financial support with postgraduate scholarships. Without their assistance I could not have undertaken this project.

DEDICATION

I dedicate this dissertation to my family.

To Richard and Doreen for instilling in me a sense of wonder about the world and a belief in the value of pursuing its mysteries.

To Joyce, Camille, and Sean for always supporting, sometimes enjoying, and occasionally enduring this part of our journey together.

FRONTISPIECE

Lateral view of a 96 hour *Strongylocentrotus purpuratus* larva, immunolabelled with the monoclonal antibody Sp12. Three mesenchymal derivatives - skeletogenic cells, blastocoelar cells, and pigment cells - are specifically labelled.



CHAPTER 1 INTRODUCTION

Development is the process whereby a single cell, the fertilized egg, is transformed into an integrated array of different cell types giving rise to a new individual. Few biological events incorporate such continuous and complex interactions as this transformation. The high fidelity of the process and the apparently spontaneous generation of new cell types and tissue forms indicates there is a detailed blueprint of development, and an exacting mechanism for realizing it. While it is certain that the blueprint is entrenched in the DNA of the fertilized egg, the mechanisms and machinery of development are largely shrouded in uncertainty. Elucidation of the molecular events underlying these enigmatic processes will yield insights into some of biology's most profound questions.

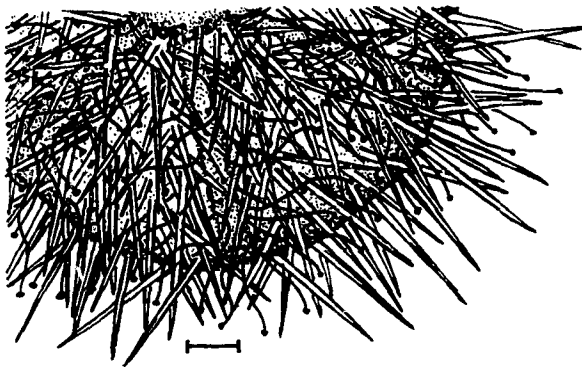
The sea urchin embryo

The study of animal development has utilized a wide variety of organisms, both invertebrate and vertebrate. Cellular slime molds, sponges, nematodes, fruit flies, sea urchins, starfish, and tunicates are intensively studied invertebrates while frogs, salamanders, chickens, and mice are well known vertebrate systems. Sea urchin embryos possess several traits that have made them one of the premier organisms for developmental studies. They are easily obtained and cultured in large numbers, and they develop rapidly, with most of their embryonic and larval development occurring within seven days of fertilization (Fig. 1; see Okazaki, 1975a for an overview of normal sea urchin development). Of particular significance for the observation of morphogenesis is their transparency, lack of a large yolk mass, and intermediate complexity. They are also well suited to biochemical and molecular biological studies as large numbers of synchronous embryos are available, cultures of a single cell type, or dissociates of a single germ layer can be isolated, transgenic individuals have been produced, and most species contain only a small

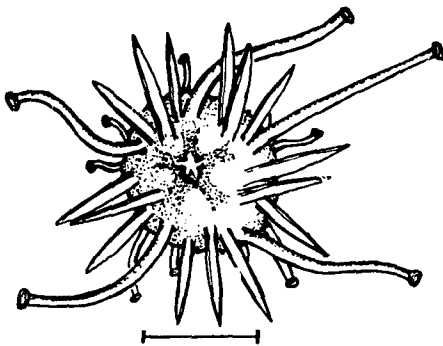
amount of yolk (which reduces interference with chemical analyses). In addition, they are deuterostomes with a regulative mode of development which makes them good models for the more complicated development of vertebrate embryos. Their positive attributes have resulted in extensive investigations of echinoid embryos over the past century. These experimental results have produced an enormous base of information one can draw on (*e.g.* Giudice, 1973; Horstadius, 1973; Stearns, 1974; Czihak, 1975; Davidson, 1986; Schroeder, 1986).

Metazoan development is a highly integrated continuum, however, analysis of the mechanisms underlying its events is simplified by splitting it into two categories, cytodifferentiation and morphogenesis. Growth is sometimes included as a third component (Trinkaus, 1984), but it plays a limited role in early sea urchin development and is not considered here.

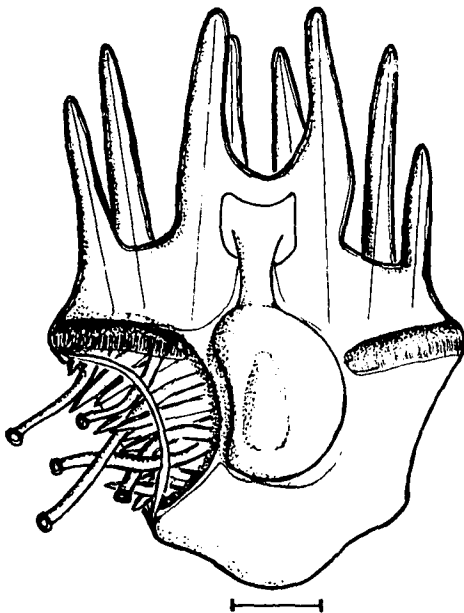
Figure 1. Life cycle of *Strongylocentrotus purpuratus*. Adults are dioecious, and fertilization is external. Upon fertilization cortical granules release their contents, elevating the fertilization envelope and forming the hyaline layer, a protective extracellular matrix (ECM) coat. The egg is mature at fertilization (*i.e.* meiosis is complete) and cleavage starts immediately with the first cell division at about two and one half hours post-fertilization (PF). Subsequent divisions occur every one to two hours (depending on temperature) and produce a hollow sphere of uniform sized, single layered cells, the blastula. The blastula hatches out of the fertilization envelope at about 24 hours PF, and swims (via cilia) into the water column. Skeletogenic mesenchyme ingress at the vegetal pole of the embryo (inferior margin in these lateral views) at about 30 hours PF. Gastrulation, with archenteron invagination, elongation, and secondary mesenchyme release, starts at about 40 hours PF and is complete by 48 hours PF. The skeleton is secreted, larval epidermis is shaped, and the gut differentiates to produce a prism larvae by 70 hours PF. Arm buds form and elongate aided by skeleton secretion, the gut matures, and coelomic pouches develop from the esophageal epithelium. Feeding on unicellular algae starts by four days PF, four distinct arms are present by seven days, and two more pairs of arms grow during the ensuing weeks. Considerable growth occurs during this planktotrophic feeding stage, and an adult rudiment forms in the left coelomic cavity. A dramatic metamorphosis occurs at about ten weeks PF. The competent larva settles on an appropriate substrate, everts its rudiment, retracts the larval arms onto its dorsum, reorganizes its digestive system, and takes up benthic existence. Metamorphosis takes less than one hour, but the histolysis and reorganization attendant on it occur over the following week. The juvenile then feeds on multicellular sessile algae and rapidly grows to sexual maturity in one to two years. Scale bars are 20 μm in the egg through pluteus, 200 μm in the metamorphic and juvenile animals, and 1 cm in the adult.



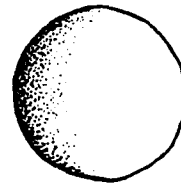
ADULT 3 yr



JUVENILE 12 wk



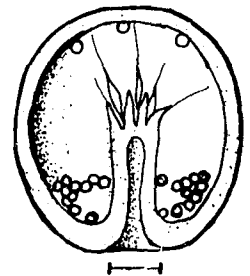
METAMORPHOSIS 10 wk



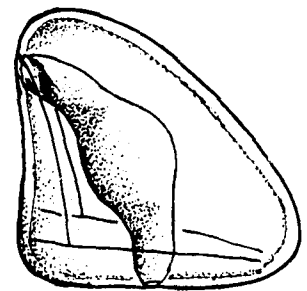
EGG 0 hr



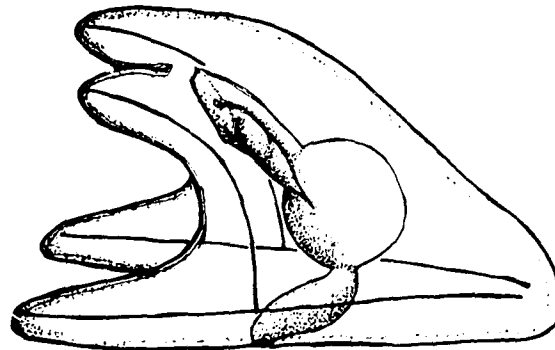
BLASTULA 24 hr



GASTRULA 45 hr



PRISM 70 hr



PLUTEUS 7 d

Cytodifferentiation

Cytodifferentiation is the acquisition, by individual cells, of the specific gene products typical of mature cells of that type. The concept of gene regulation is inherent in this definition and is fundamental to cell differentiation. A current model of differentiation envisions *trans* acting signals interacting with gene-specific *cis* regulatory sequences on the DNA to influence gene transcription. Repeated cycles of this interaction culminate in cells with widely divergent gene activity. These gene products may be either regulatory or structural, and they form the basis for cellular differentiation. While this description is very simplistic and does not reflect the many complexities now known to be involved in transcriptional regulation (Davidson, 1989), it serves as a useful model. Large numbers of structural genes are coordinately regulated in development. One proposed explanation of this phenomenon (Davidson and Britten, 1979) postulates a regulatory hierarchy utilizing repetitive DNA sequences as integrators of both transcriptional (*cis* elements common to several genes) and post-transcriptional (mRNA stabilization) regulation. This is not the only theory of coordinated gene regulation, but exploration of all those proposed is not warranted in this brief overview. Other important mechanisms of gene regulation and cytodifferentiation include chromosomal events, transcriptional events not utilizing *cis* and *trans* regulators, mRNA processing and stability, translational events, and post-translational events (Davidson, 1986; Watson *et al.*, 1987). Without discussing all these processes, it is clear that the end result of gene regulation in all its guises is the production of biological molecules, and hence processes, which characterize a given cell type.

Examination of gene products which are temporally and spatially restricted in the embryo offers potential insight into gene regulation during development. A number of such molecules have been examined in the sea urchin embryo (Davidson

et al., 1982; Angerer and Davidson, 1984). This work has included a variety of approaches. Gene expression and regulation in the histone and actin gene families has been intensively studied (reviewed in Davidson, 1986). Lineage analysis of blastomeres has been done using nucleic acid probes and monoclonal antibodies against tissue specific messages and proteins (*e.g.* the aboral ectoderm Spec proteins, Klein *et al.*, 1987, and the pigment cell surface protein labelled by Sp1, Gibson and Burke, 1985). As well, tissue specific shifts in RNA and protein production have been correlated with cell determination and tissue differentiation (*e.g.* spiculogenesis in the micromere lineage, Harkey, 1983; Harkey *et al.*, 1988). These pursuits are yielding tremendous insight into the molecular biology of sea urchin embryos and the events of cellular differentiation.

Mention must be made at this point of the inductive interactions that play a prominent role in sea urchin development. The elegant blastomere recombination experiments of Horstadius (1973 for review) have been classically interpreted by postulating a double gradient (animal and vegetal) of morphogens. Much of the molecular data gained in the past decade has been used to reinterpret Horstadius' results in more mechanistic terms (Davidson, 1989). In his model Davidson postulates that the vegetal micromeres induce, by ligand-receptor interactions at the plasma membrane, apposed blastomeres to activate gene regulatory factors. A wave of induction then ascends to the animal pole, determining successive tiers of blastomeres early in cleavage. This model is a good example of the new understanding arising from molecular studies; it also implies that there is more to development than autonomous gene regulation.

The above examples illustrate that a cell can be influenced towards its final differentiated state at numerous points on the pathway between DNA and protein. However, although cytodifferentiation has long been considered the essence of development, examination of developmental events reveals it is only part of the

process. An unorganized collection of differentiated cells does not constitute a functional embryo or juvenile organism. Differentiating cells do not exist in isolation, and to produce a viable organism they must interact with each other, and with the extracellular matrix around them. These collections of cells must undergo morphogenesis to give rise to functional, three-dimensional, species specific forms.

Morphogenesis

Morphogenesis is "the structural development of an organism or part." Pattern formation (Wolpert, 1981; Wolpert and Stein, 1984), with its mathematical and philosophical constructs (Thom, 1983) is not considered in this discussion. It is the mechanisms which generate form rather than the equations which specify it that are considered below. In animals morphogenetic processes include invagination, evagination, cleft formation, and pinching off of epithelial sheets and the delamination, migration, and reaggregation of individual mesenchyme cells. Despite the simplicity of the basic processes, morphogenesis encompasses a wide variety of developmental events. These range from the primary events of blastulation, gastrulation, and neurulation through more complex organ and appendage formation to the very complex and subtle events which result in individual body shape and visage.

An emerging paradigm for morphogenetic mechanisms in developing animals postulates interactions between cells via cell surface molecules, and between these molecules and the extracellular matrix (ECM) around them (Hay, 1981; Bissel *et al.*, 1982; Trinkaus, 1984; Edelman, 1984; Edelman, 1985; Ekblom *et al.*, 1986; McClay and Etensohn, 1987a). These interactions may give rise to preferential migration routes, establish cellular patterns, direct epithelial movements, influence epithelial folding and provide differentiation cues. Basic to an understanding of cell-cell and cell-ECM interactions (and hence, morphogenesis)

is the identification and detailed examination of the developmentally regulated molecules which mediate these interactions.

Investigations of morphogenesis in the sea urchin embryo have concentrated on the initial events of development. Blastulation, the formation of the blastodermal epithelium, has been examined, with cell - cell and cell-ECM interactions documented. Many details of this process have been elucidated with dissociation-reaggregation experiments. Mesenchyme ingression and migration have been extensively documented, both *in vivo* and *in vitro*. Gastrulation, which results in the three primary germ layers - ectoderm, mesoderm, and endoderm - has been investigated in detail. Examination of the forces which result in invagination and elongation of the archenteron has been the major thrust of these experiments. Later events such as gut morphogenesis and the formation of arms have also been documented, but not to the extent of these pre-prism transformations. Metamorphosis in sea urchins is dramatic, rapid, and extensive; it transforms the bilateral larva into a radially symmetric juvenile (Fig. 1). This aspect of development has received little investigation, but it is a morphogenetic process of impressive scope.

Fertilization of the sea urchin egg is followed by about 10 cleavage divisions over 12 to 24 hours. The fourth cleavage division at 3 to 6 hours PF gives rise to the distinctive sixteen cell embryo composed of eight mesomeres, four macromeres, and four micromeres arranged along the animal-vegetal axis. The micromeres divide asymmetrically again, and the small micromeres become coelomic cells (Pehrson and Cohen, 1986) while the large micromeres are determined at this stage to become primary mesenchyme and skeletogenic cells in older embryos and larvae. Continued cleavage results in an epithelium one cell thick, in the shape of a hollow sphere (Fig. 1). Formation of this blastula requires cell-cell and cell-ECM interactions. The hyaline layer is a thick ECM coat which is exocytosed from

cortical granules at fertilization and remains closely applied to blastodermal and epidermal cells until metamorphosis (Cameron and Holland, 1985). The hyaline layer is thought to provide the cohesive force keeping blastomeres together through early cleavage (Dan and Ono, 1952; Kane, 1973; Schroeder, 1986b). It may also play a role in mesenchyme ingression later in development (McClay and Fink, 1982; Fink and McClay, 1985). Spot desmosomes are present in the four cell embryo, and belt desmosomes have started forming then but are not fully formed until the early blastula (Spiegel and Howard, 1983). The basal lamina and hemi-desmosomes are also formed by early blastula, and these structures signal that the epithelium is formed (Spiegel and Howard, 1983). The blastocoel is not an empty space, it is filled with a gel which, it is postulated, provides support to the blastoderm and resistance to epithelial deformation in the older embryo and larva (Strathman, 1989).

Dissociation - reaggregation experiments have revealed a number of factors important to blastula formation (Spiegel and Spiegel, 1986 for review). Giudice (1962) showed that dissociated cells of several stages will reaggregate, but that pluteus-like structures only developed from early (mesenchyme blastula, early gastrula) dissociates. Species specificity, but not stage specificity has been documented in reagggregates (Giudice and Mutolo, 1970; Schneider, 1985). Homospecific binding proceeds through mutual extension of microvilli and secretion of hyaline material (Spiegel and Spiegel, 1975). Heterospecific cells will initially adhere, but neither of the above events occurs, and the cells quickly dissociate. Three very different modes of reaggregation occur in stationary cultures, dependant on the initial cell concentration (Spiegel and Spiegel, 1975), but the final pluteus-like forms are similar in all three cases. Increased percentages of normal embryos with improved symmetry are obtained when intact blastula fragments are utilized as a template for the reaggregation (Freeman, 1989). The cells from blastula

dissociates retain their apical-basal polarity (Yazaki, 1984; Schroeder, 1988) and quickly sort out once aggregated (Nelson and McClay, 1988).

Adhesion promoting factors have been identified using reaggregation assays. Butanol extracts of dissociated blastula cells or membrane preparations stimulate reaggregation (Noll *et al.*, 1979; McCarthy and Spiegel, 1983a; McCarthy and Spiegel, 1983b). The active fraction of these extracts appears to be a 22s particle, a toposome (Noll *et al.*, 1985), composed of at least six glycoproteins. While antibodies against this particle or against butanol extracts inhibit reaggregation there is some uncertainty about its cellular localization. Butanol extracts of radioiodinated cells do not contain any labelled bands on SDS-PAGE autoradiograms (McCarthy and Spiegel, 1983a), and fluorescently labelled sections show toposomes localized to the cortex of cells rather than specifically on cell surfaces (Noll *et al.*, 1985). It is also curious that toposomes can be isolated from yolk granules and that vitellogenin, a major yolk protein, appears to be the toposome precursor and also stimulates cell aggregation (Noll *et al.*, 1985; Matrangola *et al.*, 1986; Cervello and Matrangola, 1989). It may be that toposomes function through some cortical action, mediating cell surface events.

Another potent agglutinator of cells has lectin-like properties, and recognizes specific carbohydrate moieties on the surface of dissociated cells (Oppenheimer and Meyer, 1982; Tonegawa *et al.*, 1986). A stage-specific study has shown increasing sensitivity of reaggregating cells to mixed exoglycosidases with developmental age. Enzyme treated cells from hatched blastulae and gastrulae exhibit three times the reaggregation inhibition of cleavage stage cells (Watanabe *et al.*, 1982a). Further characterization of these molecules is required, but it is interesting that carbohydrate moieties appear to play a role in sea urchin cell adhesion. An exogenous lectin, concanavalin A (con A), causes dissociation of blastulae at 100 $\mu\text{g/ml}$ and inhibits gastrulation at 10 $\mu\text{g/ml}$; it also inhibits reaggregation of

dissociated cells (Lallier, 1972; Kyoizumi and Kominami, 1980; Matsumoto *et al.*, 1984).

It has long been known that calcium is essential for cell-cell adhesion in sea urchin embryos (Herbst, 1900). The hyaline layer is dissolved by calcium free seawater, and this reduces blastodermal integrity, but cell-cell adhesion also requires calcium (McClay and Matrangola, 1986). A large calcium binding protein has been isolated with calcium magnesium free seawater, and it has cell aggregating activity (Tonegawa, 1973; Tonegawa, 1984; Tonegawa *et al.*, 1986). However, the method of isolation and very large M_r of this glycoprotein indicate it may actually be hyalin (the major constituent of the hyaline layer) rather than a new cell adhesion molecule. Kondo and Sakai (1971) isolated a hyalin-like component which promoted reaggregation of dissociated cells. Similarly, a proteoglycan fraction isolated from ethylene diamine tetracetic acid (EDTA) extracts of larvae does the same thing (Akasaka and Terayama, 1984). There appears to be at least two separate mechanisms of cell adhesion utilized by reaggregating sea urchin blastula cells: an initial calcium sensitive binding is reinforced by calcium sensitive ECM (hyaline) secretion; and a butanol extractable, calcium independent binding which is also immediately apparent (McClay and Matrangola, 1986). Differential properties of cell-cell and cell-substrate adhesion during aggregation also reveal two mechanisms. The cell-cell adhesion is butanol extractable and protease insensitive, while the cell-substrate binding is protease sensitive and not extracted by butanol (Watanabe *et al.*, 1982b).

A comprehensive adhesion theory for sea urchin cells remains to be formulated, but from this brief review it is apparent that several mechanisms are likely utilized. Cell adhesion is fundamental to morphogenesis, in some cases cell adhesion molecules may even direct morphogenesis and pattern formation (Edelman, 1984; Edelman, 1986; Edelman and Gallin, 1987; McClay and Etensohn,

1987a; Bozhkova *et al.*, 1985). Cell adhesion has been linked to tissue differentiation in starfish embryos (Kominami, 1984; Kominami, 1985), and given the importance of induction in sea urchin development (see above, Davidson, 1989) adhesion may also be a factor in these embryo's differentiation. Whatever its specific functions are, cell adhesion is a fundamental prerequisite to further development.

The next morphogenetic events in the sea urchin embryo form a continuous series from primary mesenchyme ingression and migration through gastrulation proper with vegetal plate invagination, archenteron elongation, secondary mesenchyme release and dispersion, and fusion of the primitive gut to the stomodeum (Fig. 1). These events have been documented in detail with time-lapse-cinematography (Gustafson and Wolpert, 1967 for review). Examination of molecular and ultrastructural changes on the cell surface and in the ECM during gastrulation is a current endeavor, and promises new insights (McClay and Etensohn, 1987b; Spiegel and Spiegel, 1986).

The primary mesenchyme (first ingressing mesenchyme) are direct descendants of the large micromeres of the 32 cell embryo (Horstadius, 1973; Urban *et al.*, 1988). During primary mesenchyme ingression, cells at the vegetal plate undergo a number of changes, release from the blastoderm, and migrate into the blastocoel. Many of the morphological changes in ingressing cells, invaginating epithelium, and blastocoelar ECM have been documented (Akasaka *et al.*, 1980; Katow and Solursh, 1979; Katow and Solursh, 1981; Kawabe *et al.*, 1981; Amemiya *et al.*, 1982; Galileo and Morrill, 1985; Morrill and Doty, 1986; Katow and Amemiya, 1986; Amemiya, 1986; Amemiya, 1989). While some of these studies document changes in gastrulation inhibited embryos, morphological analysis can only be considered preliminary to a detailed examination of the molecular events underlying gastrulation.

Ingression occurs independent of either membrane shuttling or microtubule activity (Anstrom and Raff, 1988; Anstrom, 1989). Changes in cell surface ligands during ingression are evidenced by a reduction in the hyalin affinity of presumptive mesenchyme, and an increase in their affinity for fibronectin (McClay and Fink, 1982; Fink and McClay, 1985). Butanol extracts of primary mesenchyme (micromere derivatives) inhibit cell reaggregation, yet SDS-PAGE comparisons of this extract with reaggregation promoting epithelial cell extracts reveal little difference in molecular constituents (Smith, 1984). Mesenchyme do not appear to lose butanol-extractable adhesion molecules during ingression, but they may be segregated or modified during this event.

After ingressing, mesenchyme cells migrate on the blastodermal basal lamina. Primary mesenchyme migration has been extensively studied as a model for morphogenetic cell motility in animals (Solursh, 1986 for review). The pattern of migration and final aggregation sites appears to be mediated by the blastocoelar ECM and blastodermal cells. Injection of migration-competent mesenchyme into early blastulae results in their migration to, and accumulation at, the vegetal pole until the blastoderm and ECM have matured (Ettensohn and McClay, 1986). The ectoderm has been postulated as a template for the mesenchyme aggregates (Okazaki, 1975a for review), however, recent evidence indicates calcium-independent fibrils in the ECM direct mesenchyme movements (Amemiya, 1989). Similar ECM directed mesenchyme movements have been postulated for starfish embryos (Crawford and Chia, 1982; Abed and Crawford, 1986; Crawford, 1988).

Primary mesenchyme have been extensively investigated *in vitro*. This is in part due to their early, stable determination, which allows normal differentiation of cultured micromeres isolated at the 16 cell stage (Okazaki, 1975b) or at the mesenchyme blastula stage (Farkey and Whiteley, 1980; Mintz *et al.*, 1981). Many

aspects of differentiation and morphogenetic activity in the micromere-primary mesenchyme lineage have been reviewed in Harkey (1983) and Wilt (1987).

Cultured primary mesenchyme will migrate in a manner similar to that observed *in vivo* (Katow and Solursh, 1981). Cells deficient in sulphate or proteoglycans do not migrate (Venkatasubramanian and Solursh, 1984; Lane and Solursh, 1988), while specific ECM components stimulate migration. Fibronectin, fibronectin-collagen, and fibronectin-collagen-dermatan sulphate, in that order, promote increased levels of activity (Venkatasubramanian and Solursh, 1984; Katow, 1986). Fibronectin appears to be essential for primary mesenchyme migration (Katow and Hayashi, 1985). Synthetic peptides mimicking part of the fibronectin molecule inhibit cell migration *in vitro* (Katow, 1987). Time-lapse video studies have shown that migrating primary mesenchyme utilize thin elongated filopodia for testing the environment, anchoring to the substrate, and forming cellular connections (Karp and Solursh, 1985a; Karp and Solursh, 1985b). The filopodia appear to direct the cells' migration toward ECM deposits on the substrate (Karp and Solursh, 1985a), and toward ECM coated beads (Solursh and Lane, 1988). The cell surface ligands which mediate the molecular interactions with the ECM remain to be identified, although cell surface sulphated proteoglycans have been implicated (Lane and Solursh, 1988).

Spiculogenesis *in vitro* requires a serum supplement in the culture medium (Okazaki, 1975b; Harkey and Whiteley, 1980). Under these conditions, cultured cells undergo syncytium formation and secrete the skeleton just as they would *in vivo* (Okazaki, 1960; Gibbins *et al.*, 1969, Kerkis and Isaeva, 1984; Decker *et al.*, 1987; Decker and Lennarz, 1988). It appears that spiculogenesis requires a collagenous matrix (endogenous or exogenous) around the primary mesenchyme, but the collagen is not incorporated into the spicule matrix (Blankenship and Benson, 1984; Benson *et al.*, 1986). Carbohydrate moieties may play a role in regulating this ECM

secretion as con A and wheat germ agglutinin (WGA) bind cells to the substrate and induce secretion of ECM (Iwata and Nakano, 1984). The morphology of the spicules in culture is generally simple (Decker and Lennarz, 1988). Spicule complexity approaches normal as epithelial cells are added to the primary mesenchyme cells but normal spicules require a tight epithelium adjacent to them (Harkey and Whiteley, 1980). This effect may be a function of mesenchyme contact, as the length of time micromere descendants are cultured as aggregates is correlated with the normal appearance of the skeleton secreted (Kinoshita and Okazaki, 1984). Thus two aspects of skeletal morphogenesis, primary mesenchyme migration and spicule formation, are dependant on cell-substrate or cell-cell interactions. However, the specific cellular molecules involved in these processes are not known.

Gastrulation is a dramatic process, involving both epithelial deformation and mesenchymal cell traction and release (Gustafson, 1975). It is divided into two stages, primary invagination which includes the initial indenting of the flat vegetal plate, and secondary elongation of the archenteron across the blastocoel. Secondary mesenchyme (the second set of mesenchyme ingressing) are released during primary invagination (chromogenic or pigment cells - Gibson and Burke, 1985) and during elongation (blastocoelar cells or classical secondary mesenchyme).

A number of theories have been proposed to explain the initial invagination of the vegetal plate. Gustafson (1969) stressed epithelial dynamics in his theory of gastrulation, with cell rounding and ECM restraint causing invagination. However, cells do not change shape much, and only the vegetal one half to one third of the embryo is required for primary invagination (Ettensohn, 1984). The potential role of regionalized cell division has been described (Nislow and Morrill, 1988), although experiments with aphidocolin appear to rule out cell division as a driving force in gastrulation (Stephens *et al.*, 1986). These experiments and theories are based on the mechanical properties of the invaginating epithelium. Molecular factors at the

cell surface may contribute to mechanical force through selective adhesion to ECM and adjacent cells (Myers, 1990; McClay *et al.*, 1987).

Archenteron elongation is probably driven by two processes - initial rearrangement of cells in the archenteron epithelium (Ettensohn, 1985; Hardin and Cheng, 1986; Keller and Hardin, 1987) followed by traction and guidance provided by the secondary mesenchyme at the tip of the archenteron (Dan and Okazaki, 1956; Spiegel and Burger, 1982; Hardin, 1988).

The search for molecular factors underlying gastrulation has identified potential candidates based on both temporal and spatial restriction of their appearance. Polyclonal antisera against different stages of embryo reveal changes in antigens through development (Westin, 1972). Greater resolution with two dimensional polyacrylamide gels has resolved several proteins which change in abundance during gastrulation (Bedard and Brandhorst, 1983), and gels of cell fractions show some discreet differences (Harkey and Whiteley, 1982; Pittman and Ernst, 1984). Newly expressed antigens, specific to the cell surface, are detectable in gastrula stage embryos (McClay *et al.*, 1977; McClay and Chambers, 1978; McClay, 1979). Spatially restricted cell surface antigens are specifically localized to endoderm, mesoderm and ectoderm following gastrulation (McClay and Marchase, 1979). Glycoprotein production is also varied during gastrulation (Carson *et al.*, 1982; Lennarz, 1983; Lennarz, 1986) through mRNA production (Lau and Lennarz, 1983), and through regulation of the glycosyltransferases required for their production (Welply *et al.*, 1985). These molecular changes tantalize, but lack the precision required for an understanding of developmental mechanisms.

Lectin localizations have shown more details of these glycoprotein changes. There is a decrease in con A agglutinability of dissociated cells between 24 and 72 hours (Krach *et al.*, 1974). Con A localizes to the whole blastodermal basal lamina in mesenchyme blastulae, but is restricted to the animal half of the embryo in mid-

gastrulae (Katow and Solursh, 1982; DeSimone and Spiegel, 1986a). Lectin binding differences between epithelial and mesenchymal cells have been noted (Kew, 1984). Ingressed primary mesenchyme are not labelled by con A, but are labelled by WGA (DeSimone and Spiegel, 1986a; DeSimone and Spiegel, 1986b; Etensohn and McClay, 1987).

Spatial and temporal localization of ECM and cell surface molecules with antibodies and radiolabelling has revealed, in greater detail, tissue specific proteins and glycoproteins. Numerous ECM components have been identified in the blastocoel of the sea urchin embryo (see Spiegel *et al.*, 1989 for review). Some of these components have complex patterns of both apical and basal secretion from blastodermal cells (Alliegro and McClay, 1988). Fibronectin has been identified in the basal lamina, in the blastocoel, and on cell surfaces (Spiegel *et al.*, 1980; Spiegel *et al.*, 1983; Katow *et al.*, 1982; DeSimone *et al.*, 1985). This is of particular importance given fibronectin's role in mesenchyme migration *in vitro*. Laminin is localized to blastodermal cells and basal lamina, but not to mesenchyme cells early in gastrulation (Spiegel *et al.*, 1983; McCarthy and Burger, 1987). Echinoderm laminin is immunologically and ultrastructurally similar to the vertebrate form (McCarthy *et al.*, 1987). A novel ECM molecule, echinonectin, is tightly associated with cells but on the apical hyaline layer side, not in the blastocoel (Alliegro *et al.*, 1988). A microfibril ECM component, similar to that associated with elastin in vertebrates, forms a meshwork in the blastocoel and is strikingly labelled with the monoclonal antibody Sp14 (Burke and Tamboline, 1990). Types I, III, and IV collagen are immunologically localized to the blastocoelar ECM (Crise-Benson and Benson, 1979; Benson and Sessions, 1980; Wessel *et al.*, 1984). Radioactive proline, utilized to identify collagen-like proteins, localized these proteins to the archenteron during gastrulation, the ectoderm in prism stage larvae, and to the spicules in plutei (Mizoguchi *et al.*, 1989). Antisera and radiolabelling have also provided evidence of

dermatan sulphate, heparan sulfate and chondroitin sulfate in the blastocoelar ECM (Solursh and Katow, 1982; Wessel *et al.*, 1984).

Stage specific cell surface antigens have been identified and characterized with high levels of precision using monoclonal antibodies. Msp130 is a primary mesenchyme cell surface glycoprotein thought to play a role in calcium metabolism (Carson *et al.*, 1985). Mesenchyme express this antigen at the time of ingression (Anstrom *et al.*, 1987; Leaf *et al.*, 1987; Farach *et al.*, 1987; Decker *et al.*, 1987). Another primary mesenchyme antigen has similar properties to msp130, and may be a species' variant (Shimizu *et al.*, 1988). A large M_r antigen, Meso 1, is expressed on the surface of both primary and secondary mesenchyme at the time of ingression, and appears to form a component of the blastocoelar ECM in older embryos (Wessel and McClay, 1985). A 110K cell surface antigen is specifically localized to chromogenic mesenchyme as it becomes motile (Gibson and Burke, 1985; 1987). A number of cell surface antigens with discrete localizations on the ectoderm and archenteron are expressed or change their pattern of expression during gastrulation (McClay *et al.*, 1987). Endo16 is an endoderm and secondary mesenchyme specific cell surface protein which appears to contain an RGD sequence (Nocente-McGrath *et al.*, 1989).

Identified ECM components likely have functions analogous to their role in vertebrates. Msp130 is probably involved in skeleton deposition, Endo16 may link cells to the ECM, and Meso1 and the pigment cell antigen are loosely implicated in cell migration. However, as indicated, the developmental role of most of these antigens is only tentatively established or remains unknown.

A number of strategies have been employed to disrupt development by interfering with specific molecular groups. Sulphate deprived embryos do not gastrulate, and their mesenchyme does not migrate after ingressing (Herbst, 1904; Runnstrom *et al.*, 1964; Immers and Runnstrom, 1965; Karp and Solursh, 1974;

Katow and Solursh, 1981; Akasaka and Terayama, 1983; Lovtrup-Rein and Lovtrup, 1984). Likewise, tunicamycin - an inhibitor of N-linked glycosylation - blocks mesenchyme migration, although cells ingress normally (Schneider *et al.*, 1978; Heifetz and Lennarz, 1979). When proteoglycan synthesis is inhibited with aryl- β -D-xyloside, sodium selenate, or 2-deoxy D-glucose, morphogenesis beyond the blastula is similarly inhibited (Kinoshita and Saiga, 1979; Solursh *et al.*, 1986). Other factors, including blastocoelar collagen (Butler *et al.*, 1987; Wessel and McClay, 1987) are required for mesenchyme migration and gastrulation.

Injection of lectins and proteases into the blastocoel of gastrulating embryos causes retraction of the secondary mesenchyme filopodia and regression of the archenteron. Apparently, the crosslinking of carbohydrate moieties prevents the required molecular or physical interactions (Spiegel and Burger, 1982). Lectins, however, can bind a number of different molecules, which makes them less specific reagents, and makes it difficult to identify key components. In contrast, monoclonal antibodies, in general, label a single molecular species; this makes them excellent reagents for the identification and perturbation of individual components which may be crucial in development.

The binding of ECM components with antibodies has provided a number of interesting results. Early in development a monoclonal antibody against a vitelline layer antigen inhibits fertilization (Gache *et al.*, 1983). Disruption of laminin with specific antibodies, has shown that it mediates blastodermal cell shape changes during gastrulation (McCarthy and Burger, 1987). A monoclonal antibody directed against a blastocoelar matrix of microfibrils appears to interfere with secondary mesenchyme migration when injected into the blastocoel, although this effect is overcome with continued development (Burke and Tamboline, 1990). A monoclonal antibody against a blastocoelar fibronectin-binding acid polysaccharide induces multiple or deformed spicules when microinjected into blastulae (Iwata and

Nakano, 1985a). In culture this same antibody enhances spicule formation and calcium uptake (Iwata and Nakano, 1985a; Iwata and Nakano, 1985b). An anti-hyalin monoclonal antibody perturbs morphogenesis, especially gastrulation, when embryos are simply cultured in it (Adelson and Humphreys, 1988). Another monoclonal, which binds one component of the apical lamina (part of the hyaline layer), dramatically yet reversibly prevents gastrulation when embryos are cultured in it (Myers, 1990).

A number of anti-ECM antibodies disrupt development, but few published studies have used anti-cell-surface-molecule reagents. The anti-Meso 1 monoclonal antibody temporarily prevents migration of mesenchyme when it is injected into the blastocoel, however, this result has not been documented in detail (McClay *et al.*, 1987; McClay and Etensohn, 1987). The monoclonal antibody 1223, which binds msp130 reduces calcium uptake and spicule formation by mesenchyme *in vitro* (Carson *et al.*, 1985) but no effects have been documented *in vivo*.

Continuing with the remainder of post-gastrulation development, fusion of the archenteron to the stomodeum and formation of the mouth has been described by Gustafson and Wolpert (1963). This event has not been investigated in any detail, but presumably specific cell adhesion molecules and epithelial-ECM interactions are required (McClay and Etensohn, 1987b; McClay *et al.*, 1987). This potentially revealing event awaits further investigation.

The morphogenetic changes involved in gut maturation are readily observed in the transparent sea urchin embryo. The simple tube lengthens, and swells in diameter except at the sites of two sphincters: the cardiac between the esophagus and stomach, and the pyloric between the stomach and intestine. The forces underlying these events are probably mediated by growth of the archenteron and by cytoskeleton constriction (Burke, 1980a; Burke and Chia, 1980). Expression of tissue-specific antigens in the gut does occur at this time (McClay *et al.*, 1987; Burke,

pers. comm.). The timing and localization of these antigens' expression indicates they may be morphogenetic ligands or secretory molecules required for normal gut function.

As the gut takes on its differentiated form, the ectoderm of the embryo also changes. The body outline of the animal is first prism shaped, then pluteus shaped as the larva grows arms (Fig. 1). These changes appear to result from a combination of forces - those intrinsic to the ectodermal epithelium, and those mediated by skeletal rod growth. Mesenchyme's role in these shape changes may involve more than simple deposition of the skeleton and the ensuing pressure it exerts. There may be some inductive role of the mesenchyme adjacent to the epidermal arm buds (Horstadius, 1975; Gustafson and Wolpert, 1961). There is also a possibility that the secondary mesenchyme play some role in these shape changes. A network of multipolar mesenchyme underlie the ectoderm, and may provide structural tension to the epithelium as it is deformed during prism and pluteus formation.

The final, and most dramatic morphogenetic event of the sea urchin larva is metamorphosis. Upon maturation and receipt of the appropriate cue, larvae settle onto the substrate, evert the adult rudiment, and lose most of their larval organs and appendages (Fig. 1) (MacBride, 1903; MacBride, 1914; Hyman, 1955). Many of these structures are retained in the juvenile's body cavity and histolysed or otherwise reorganized for adult functions (Chia and Burke, 1978; Cameron and Hinegardner, 1978). While some aspects of metamorphosis have been examined in detail, much of it has only been described from microscopic observation. Arm retraction is known to be actin mediated (Cameron and Hinegardner, 1978; Burke, 1985), and gut reorganization is conservative, retaining most of the larval structure (Chia and Burke, 1978; Burke, 1978a). Some epidermal tissues are also retained (Cameron and Hinegardner, 1978; Cameron and Holland, 1985). Studies of the gene products shared by larvae and adults have also been done (Galau *et al.*, 1976; Richardson *et*

al., 1989; Drager *et al.*, 1989). Despite these investigations, nothing is known of the molecules mediating the profound reorganization of metamorphosis.

Many of the molecular investigations relevant to sea urchin morphogenesis have been reviewed above, and it is clear that morphogenetically active molecules are likely to be found on the cell surface or in the ECM (Yamada, 1983). While it is certain that some intracellular molecules will also prove to be of fundamental importance - the cytoskeletal proteins would be in this category - they still must interact with the cellular environment, probably through integral plasma membrane molecules. Cell - environment interactions remain the ultimate control interface in animal morphogenesis and differentiation (Wessells, 1977).

Summary

In summary, the search for, and discovery of, morphogenetically active molecules in the ECM and on cell surfaces in the sea urchin embryo is yielding new insights into the mechanisms of development. Several cell adhesion and ECM components have been identified in sea urchins. These classes of molecule have been shown to be involved in fundamental morphogenetic processes of other species (Edelman and Gallin, 1987; Takeichi, 1988; Fristrom, 1988); the sea urchin embryo is an ideal animal in which to extend these observations. Cell - ECM interactions in sea urchin mesenchyme migration is readily investigated; it has and will yield insights into this developmental phenomenon. The mechanics and cellular signalling attendant on gastrulation are also being dissected at the molecular level in sea urchin embryos. These processes are fundamental to all animal development and their elucidation in sea urchins will provide a detailed model for other organisms. Later events in larval development and metamorphosis offer a complex set of interactions, which have only begun to be investigated. Much is known about the development of this model organism, but many questions remain.

Experimental rationale

Among the significant questions facing sea urchin embryologists is the identification and characterization of cell surface molecules involved in morphogenetic events. Molecular mechanisms in development will be elucidated using molecular methods. The method chosen in this project was to generate monoclonal antibodies directed against tissue specific cell surface antigens in the sea urchin embryo. Such antibodies allow characterization of the labelled antigens, and can potentially disrupt morphogenetic events. Antibody experiments can thus provide information on specific molecules, and their roles in development. Tissue specific antibodies can also identify cell lineages and document cellular differentiation.

The remainder of this dissertation provides the results of my endeavors to do this. Chapter 2 outlines the production, purification, and manipulations of the monoclonal antibody Sp12. Chapter 3 documents the localization of the Sp12 antigens from egg through to adult. Chapter 4 provides biochemical details about the antigens and epitope recognized by Sp12. Chapter 5 is a detailed description of blastocoelar cells - a prominent secondary mesenchyme derivative. Chapter 6 describes the antibody-mediated disruption of embryos and dissociated cells. Chapter 7 offers a summary of my findings and an indication of the directions research utilizing Sp12 could go in the future.

CHAPTER 2 MONOCLONAL ANTIBODY PRODUCTION

INTRODUCTION

The technique of plasma cell - myeloma fusion to produce immortal, monoclonal cell lines secreting a single species of antibody (Kohler and Milstein, 1975) has revolutionized immunological methods. Monoclonal antibodies have great specificity and allow a precision of biological molecule identification in biological systems previously unknown. Such specificity allows a reductionist approach to complex biological systems, by dissecting out epitopes and antigens one by one. The range of projects which have utilized monoclonal antibodies is enormous, and they offer many advantages over polyclonal sera, however, these powerful reagents are not free of problems.

Specificity for a single epitope is an important attribute of these reagents. Production considerations offer two more advantages of note. Highly specific clones can be generated from crude immunogen, as specificity comes with cloning after the fusion. Then, once the hybridoma cell line is stable, there is no limit on the amount of antibody (and it is always the same antibody) that can be produced by this immortal source. In addition to the identification and characterization of biological molecules, monoclonal antibodies can interfere with the function of these molecules in a very selective way (Milstein and Lennox, 1980). Because they bind a single epitope, any disruption of function can be interpreted with much greater precision. The potential for elucidating function promises tremendous insight into processes analysed with these molecular probes.

The high degree of specificity of monoclonal antibodies may be a disadvantage as small epitopes can be shared by a number of different antigens. Such redundancy would eliminate the advantages of monospecificity. In addition, since only one antibody is present each clone has a single affinity (which may not be

very strong) peculiar to that antibody (Goding, 1986). Polyclonal antisera contain an array of antibodies with different epitopes and affinities, which may give better labelling of an antigen. In fact, redundant epitopes may be viewed as an opportunity, indicating a degree of homology between the labelled antigens. Affinity difficulties can be overcome by stringent selection criteria or by using panels of monoclonal antibodies. The problems with monoclonal antibodies are far outweighed by their advantages.

Monoclonal antibodies against tissue specific antigens were generated as a first step in this project. One of these antibodies, Sp12, was selected and used for all subsequent studies. This chapter outlines the production, cloning, purification, and generation of Fabs of Sp12.

MATERIALS AND METHODS

Sea urchin embryonic and larval culture

Larval culture followed the procedures of Strathman (1968). *Strongylocentrotus purpuratus* were collected at Point No Point, B.C. Gametes were obtained by injection of 0.55 M potassium chloride into the body cavity. Eggs were shed into a beaker of seawater filtered with a Whatman No.1 paper filter (FSW) and sperm was collected dry - undiluted from the top of a dry animal - in a pasteur pipette. Eggs were passed through a 102 μ m Nitex filter to eliminate spines and debris, and were washed twice in FSW. Eggs were fertilized at a concentration of about 5000/ml in 600 ml beakers with two to four ml of a 0.1% suspension of sperm. Embryos were grown at 12°C to 14°C in FSW; as a monolayer in shallow culture dishes until hatching, and in two liter beakers thereafter. Densities ranged from 1000 embryos/ml for young cultures to 5 larvae/ml for older cultures. After four days post-fertilization (PF) larvae were fed unicellular algae daily, *Dunelliella salina* or a Tahitian strain of *Isochrysis*, and the water was changed twice a week.

For screening of culture supernatants and antibody localization studies (Chapter 3) larvae were fixed for three to four hours at 21°C with 4% paraformaldehyde in FSW. They were rinsed three times in FSW and stored at 4°C in FSW with 0.1% sodium azide added as a preservative. Larvae were permeabilized by extraction with acetone (-20°C, 3 min), or 0.6% Triton X-100 in FSW (21°C, 30 min), then rinsed in FSW, and used immediately in the assay.

Immunization

Immunogen preparation and inoculation schedules were as described in Burke and Gibson (1986). Immunogen was a 1:1 combination of fixed pluteus extract and adult test extract. Plutei were prepared by lysing them in distilled water (DW) (0°C, 30 min), dounce homogenizing, then centrifuging (4000g, 10 min). The supernatant was discarded and pellets fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, Dulbecco's formula) (21°C, 1 hr). Adult test was prepared by removing all spines and soft tissue and homogenizing the cleaned test in a waring blender in 10 ml of PBS. The suspension was centrifuged (2100g, 10 min) and the supernatant and cellular debris overlying the calcium carbonate pellet were fixed as above. Typically, four female BALB/c mice, four to six weeks old, were inoculated three times, at biweekly intervals. Each received 0.2 ml subcutaneous and 0.3 ml intraperitoneal injections of immunogen emulsified in an equal amount of Freund's adjuvant (complete for the first inoculation, incomplete thereafter). Mice were given a final booster intravenously, without adjuvant, four days before the fusion.

Prior to fusion serum titres of anti-sea urchin antibodies were determined using indirect immunofluorescence on fixed plutei (see below). This technique, while not quantitative, allowed a crude but rapid comparison of each mouse's immune response. The mouse with the highest titre was sacrificed for the fusion.

Fusion

Two fusion protocols were utilized in this study, however, only the second produced hybridomas. The first is outlined here for completeness, and to allow comparison of the techniques. The original fusion procedure followed Galfre and Milstein (1981). Briefly, splenocytes and myeloma cells were centrifuged together into a pellet, then 1 ml of 50% polyethylene glycol (PEG) in RPMI tissue culture medium (Gibco) was added dropwise with stirring over one minute. The pellet was stirred a further two minutes, then the suspension was diluted out with RPMI. The cells were washed once, then aliquoted into two 24 well tissue culture plates (Falcon) for hypoxanthine-aminopterin-thymidine (HAT, Sigma) selection of hybridomas. This procedure resulted in very poor hybridization rates, so a second protocol was utilized.

The second fusion protocol was a modification of Gefer *et. al.* (1977). The spleen from a high titre mouse was harvested aseptically, and 10^8 nucleated cells were combined with 10^7 NS-1 myeloma cells in serum-free RPMI. The cells were centrifuged (400g, 5 min) and the pellet was resuspended in two ml of 35% PEG in RPMI at 21°C. The suspension was centrifuged (200g, 2 min) then let stand at 21°C until a total of 8 minutes had elapsed. Supernatant PEG-RPMI was removed and the pellet was resuspended in 40 ml of serum-free RPMI at 37°C. The cells were centrifuged (400g, 8 min) and resuspended in 100 ml of 20% fetal calf serum (FCS, Hyclone) in RPMI with 10^5 splenocytes/ml as feeder cells. This suspension was plated out at two ml per well in two 24-well plates, and incubated at 37°C with a 5% carbon dioxide atmosphere. After one day, one ml of supernatant was removed from each well and replaced with one ml of double concentration HAT in 20% FCS-RPMI. After one to two weeks all the unfused cells were dead and HAT was replaced with HT in subsequent feedings. Cells were fed at four day intervals by removal of one ml of medium and replacement with one ml of fresh medium.

Growth positive wells were screened for sea urchin larvae specific antibodies using indirect immunofluorescence on fixed and permeablized whole mount plutei (Burke and Gibson, 1986). Briefly, mac-tac masks with 12 holes punched in them were stuck to glass slides and permeablized plutei were adhered to each assay well with poly-L-lysine. Fifteen μ l of supernatant (or mouse serum) were placed on top of each assay well and incubated (21°C, 1 hr). Slides were washed three times in PBS and 10 μ l of fluoresceine isothiocyanate conjugated rabbit anti-mouse secondary antibody (FITC-RAMIG, Sigma) diluted 1:50, were applied and incubated (21°C, 30 min). After washing specimens were mounted in 50% glycerol in PBS with 30 mM n-propyl gallate added to reduce bleaching of the fluorescence. Plutei were examined using epifluorescence on a Zeiss Universal microscope.

Hybridomas secreting tissue specific antibodies were grown up in culture flasks and cloned or frozen in liquid nitrogen for future investigation (Goding, 1986). Cells were frozen by suspending 10^7 hybridomas in one ml of 90% fetal calf serum with 10% dimethyl sulphoxide, and sealing the suspension in a 1.5 ml cryotube (Corning). The tubes were frozen by incubation overnight in styrofoam jackets surrounded by dry ice. The following day tubes were placed in a liquid nitrogen container for long term storage at -190°C.

Cloning and ascites production

Cell lines secreting pluteus specific antibodies were cloned three times by limiting dilution to ensure monoclonality (Goding, 1986). Antibodies in cloned hybridoma supernatants were isotyped using a sandwich enzyme-linked immunosorbent assay (ELISA). The assay utilized an anti-mouse-immunoglobulin trapping antiserum, and subclass specific anti-mouse-antibodies conjugated to horseradish peroxidase (HRP) (Caltag).

Supernatants were utilized in some procedures, and were produced by allowing the cells to overgrow in the culture until the medium was yellow, and the

cells had started to die. Cells and debris were centrifuged out (800g, 10 min) and the supernatant was stored at 4°C with 0.1% sodium azide. Ascites fluid was produced by injecting pristane primed BALB/c mice with 10^7 hybridoma cells intraperitoneally. Ascites was harvested by daily tapping of swollen mice with an 18 gauge hypodermic needle. Alternatively, swollen mice were sacrificed, the abdominal cavity opened, and fluid and cells removed aseptically with a pasteur pipette. After centrifuging out cells and debris (800g, 15 min) ascites fluid was stored, neat or diluted 1:3 in glycerol, at -20°C.

Primary monoclonal antibodies used in this study were Sp12 - the twelfth cloned antibody produced in our lab against *Strongylocentrotus purpuratus*, and NRH - a monoclonal antibody against the plant enzyme ribulose biphosphate carboxylase, secreted by a non-relevant hybridoma (at least for this study the hybridoma was non-relevant).

Antibody Purification

Antibody was purified on a 25 ml SP-Trisacryl M (LKB) cation exchange column (Boonekamp and Pomp, 1986). Ascites fluid was diluted 1:20 in 50 mM sodium acetate, pH 5.5 and run onto the column at 30 ml/hour. After washing for two hours with this buffer, antibodies were eluted with 50 mM sodium acetate, 1.0 M sodium chloride, pH 5.5, and came off as a sharp peak about 50 min after addition of the elution buffer. Peak fractions were collected, pH adjusted (to about 7.0), pooled, and dialysed into FSW.

Alternatively, antibody was purified from supernatants. Clarified supernatant from 50 ml cultures was diluted 1:9 in the 50 mM sodium acetate buffer, and run onto the column at 60 ml/hour. After washing as above the bound immunoglobulins were eluted with a 0 to 0.3 M linear sodium chloride gradient, and collected in 5 ml fractions with immediate pH adjustment. Fractions in the main elution peak were pooled and immunoglobulins were precipitated by dropwise

addition of ammonium sulphate to a final concentration of 50%. The precipitate was centrifuged down (10,000g, 15 min), resuspended in 0.22 μ m millipore filtered sea water (MFSW), then dialyzed against FSW.

While the ion exchange column enriched samples in antibody, a number of contaminating proteins remained and a third method of purification was employed to obtain a purer preparation of immunoglobulins. Protein A-sepharose (Sigma) was used to isolate immunoglobulin G from ascites fluid. Immunoglobulins were precipitated from ascites fluid by ammonium sulphate, as above. The precipitate was centrifuged (10,000g, 15 min) and dissolved in 0.1 M phosphate buffer (pH 8.2). After dialysing against phosphate buffer for 24 hours, the solution was run over a 5 ml protein A-sepharose column at 20 ml/hour. The run-through was collected and run over the column a second time to ensure a high degree of IgG extraction. The column was washed for one hour with phosphate buffer and IgG was eluted with 0.1 M citrate buffer at pH 3.5. Two ml fractions were collected, and the pH was immediately adjusted to about 7.0. The column was regenerated by washing with 0.1 M citrate buffer (pH 2.5) for one hour, followed by phosphate buffer for at least one hour. Before use in live preparations, the antibody was dialyzed into FSW, and for Fab production it was dialysed into 10 mM Tris-HCl (pH 7.2) with 0.2 mM EDTA.

In all three cases, final antibody solutions were assayed for tissue specificity, run on polyacrylamide gel electrophoresis (PAGE) to determine purity, and had their protein concentration determined (Bradford, 1976). Active fractions of similar purity were pooled, aliquoted and stored frozen at -20 °C.

Fab fragment production

Protein A purified IgG was dialysed into 10 mM Tris-HCl (pH 7.2), 0.2 mM EDTA. After 24 hours dialysing the sample was vacuum centrifuged to concentrate it ten-fold. The protein concentration of the sample was determined (usually 0.5 -

1.0 mg/ml), dithiothreitol (DTT) was added to a final concentration of 0.1 mM, the pH was adjusted to 7.2, and papain was added at 1/100 the protein concentration of the sample (5 - 10 μ g/ml). Papain was activated by preincubation in Tris/EDTA/DDT buffer for 15 minutes at 37°C. The solution was incubated for 2 hours at 37°C and the reaction was stopped with iodoacetamide (25 mg/ml final concentration). The reaction mixture was dialysed into 0.1 M phosphate buffer (pH 6.5), eliminating free iodoacetamide, and Fab and Fc fractions were separated on the protein A column. Fab fragments in the run through from the column were collected, assayed for tissue specificity and purity as above and pooled. Fab fragments were dialyzed against FSW and their concentration determined by protein assay. Fc and undigested antibody were eluted from the column with citrate buffer (pH 3.5) and retained for assay of purity and tissue specificity.

RESULTS

Immunization, fusion, and cloning

Immunized mice all produce anti-pluteus antibodies, although there are variations in the immunofluorescence of each serum in pre-fusion assays. The sera do not have any discernable tissue specificity, but high levels of hyaline immunoreactivity could mask visualization of labelled cells or tissues within the larvae. For each fusion the mouse with the highest overall level of serum immunoreactivity was sacrificed, and its spleen utilized in the procedure.

None of the fusions utilizing Galfre and Milstein's (1981) procedure produced viable hybridomas. Fusion rates were consistently very low, and no clones of cells secreting anti-sea urchin antibodies were recovered. After several fusions, during which different aspects of the procedures and media used were altered, this protocol was abandoned and a new procedure employed.

The fusion procedure of Gefter *et al.* (1977) greatly increased the number of viable hybridomas produced each fusion. The fusion which produced Sp12 is a typical case. After two weeks 21 of the 48 wells were positive for growth, and 8 of these had detectable anti-pluteus antibodies in their supernatant. After three weeks 13 wells were positive for antibody, and after four weeks 18 wells contained detectable anti-pluteus antibodies. The Sp12 immunoreactivity appeared after three weeks, and was unique - no other wells contained antibodies with this pattern of tissue specificity, labelling pigment cells, blastocoelar cells, skeletogenic cells, and skeletal rods (see frontispiece and Fig. 2).

One day after discovery of the Sp12 cell line, it was cloned by limiting dilution in one 96 well plate at 0.5 cells/well. Two weeks later 7 wells out of 96 were positive for growth, and 4 of those were positive for Sp12 immunoreactivity. After ensuring positive wells had only a single plaque of cells, they were passaged to 24-well plates, then culture flasks for recloning and freezing.

The second cloning of Sp12 was done in two 96 well plates at two cells/well. Twelve days later most of the 192 wells were positive for growth. Only wells which contained an unambiguous single plaque of cells were assayed. Of 57 such wells, 55 were positive for Sp12 immunoreactivity. Cells from selected wells were cultured as above, for recloning and freezing.

To ensure monoclonality the cell line was cloned a third time. Cells were plated out at one cell/well in two 96 well plates. After two weeks 50 wells were positive for growth and 48 were positive for Sp12. The well with the highest level of immunoreactivity was selected for use in all further studies. Using the nomenclature of Pearson *et al.* (1977) the final clone is Sp12/3.15.12.

All positive supernatants have had the same tissue specificity through all phases of cloning, and in the last two clonings more than 95% of the wells positive for growth were positive for Sp12 immunoreactivity. All of the cell lines grown up

from frozen storage over three years have maintained the tissue specificity of Sp12. Similarly, when cells are grown in continuous cell culture for up to two months they do not change tissue specificity or apparent antibody titre in exhausted supernatants.

The supernatants were isotyped using subclass-specific antibodies. While levels of IgM are high in the Sp12 sample, this is non-specific and common to most supernatants examined. Sp12 is an IgG2b and NRH is an IgG1.

Antibody production, purification, and Fab generation

Culture supernatants were used in the initial characterization of the antibody's tissue specificity. Clarified supernatants stored with preservative at 4°C are stable for about three months, after which time antibody titre appears to drop. To obtain large amounts of antibody without contaminating serum immunoglobulins 50 ml cultures of cells were grown and harvested in a similar manner.

Ascites mice were either tapped over three or four days, then sacrificed, or sacrificed without tapping for a single surgical harvest of the ascites fluid. Tapped mice typically yield five to six ml of fluid and cells, while single harvest mice provide three to four ml. The fluid contains about 20% cells by volume which was removed before storage. Glycerol diluted ascites does not freeze at -20°C and remains immunoreactive for at least two years despite repeated use of the same vial. Although ascites contains a mixture of nonspecific serum immunoglobulins, it provides a much higher concentration of specific antibody than tissue culture supernatant and was used almost exclusively in all immuno-procedures.

Antibody was purified from both ascites and tissue culture supernatant. Purity of the preparations varies. Protein A purified ascites appears the cleanest on silver stained gels with only two contaminating bands, both of low abundance, at 60K and 85K. Cation exchange purified ascites and supernatant both contain about 12 contaminating bands, several of which are as abundant as the antibody bands. Purified supernatant has the advantage of containing predominantly the hybridoma

immunoglobulin, although there could be a small contribution from the fetal calf serum. Purified antibody from ascites contains large quantities of contaminating non-specific serum immunoglobulins which can not be separated from the hybridoma produced antibody. This mixture may include IgM, IgA, and all IgG subclasses in ascites purified by ion exchange, but should be restricted to IgG subclasses in the protein A purified preparation. Final protein concentrations of purified antibody solutions varied from 0.43 mg/ml (in 15 ml of buffer) to 1.31 mg/ml (in 5 ml of buffer) (ascites over cation exchange column) to 1.63 mg/ml (in 3 ml of buffer) (supernatant over cation exchange column, and concentrated before dialysis into FSW) to 0.11 mg/ml (in 10 ml buffer) (ascites over protein A column). Based respectively on these recoveries antibody concentration in ascites ranged from 5.3 mg/ml to 3.2 mg/ml to 1.2 mg/ml.

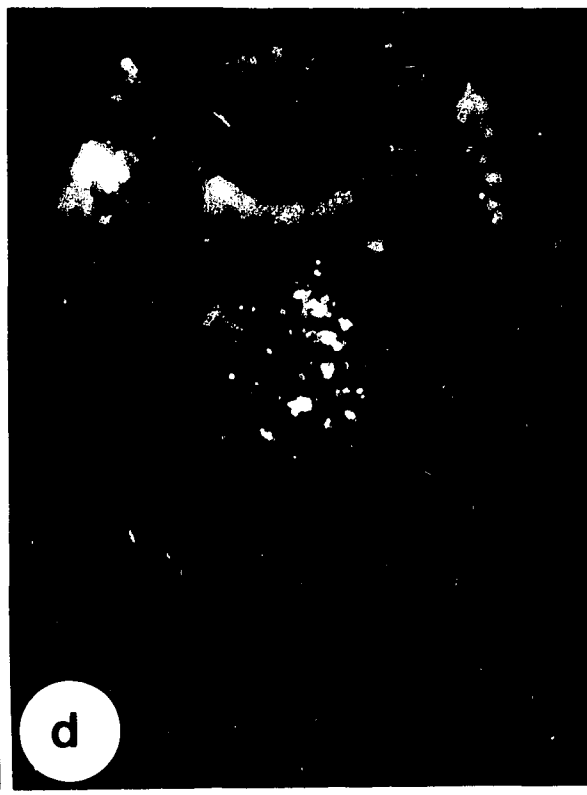
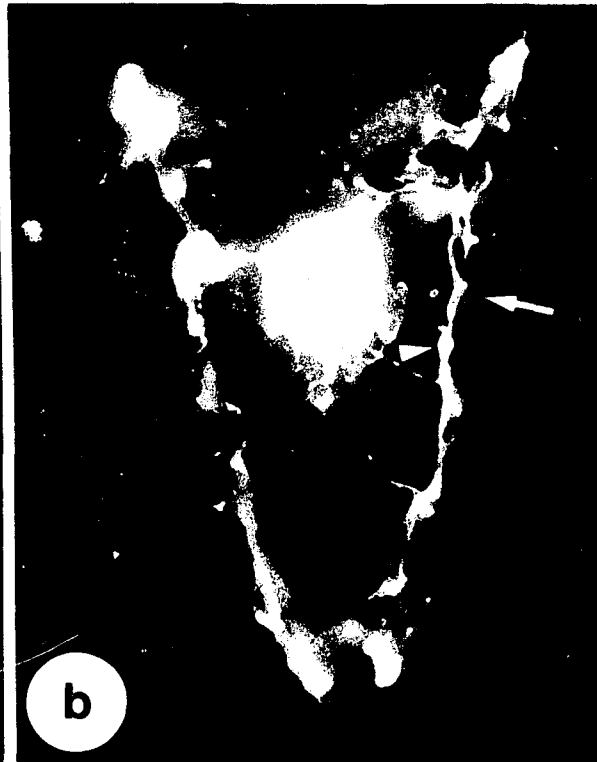
Tissue specificity

The tissue specificity of Sp12 is documented in Chapter 3, however, some mention of it is made here to introduce Sp12 labelling and to illustrate an unusual dose dependant change in the antibody's localization. Three mesenchymal derivatives are labelled: skeletogenic cells, which secrete the larval spicules; blastocoelar cells, which appear to be a connective tissue component in the blastocoelar space of the larva; and pigment cells in the epidermis of the larva. Skeletal rods as well as the cells which secrete them are immunoreactive. While this general pattern of immunoreactivity is invariant, there are considerable differences in the intensity of immunoreactivity of individual larvae, and individual cells within larvae. In some larvae pigment cells are only occasionally immunoreactive, while in others all pigment cells are intensely labelled. Similarly, some larvae have prominent immunofluorescent blastocoelar and skeletogenic cells while others have only a low level of labelling. Even within one larva, immunoreactivity may be uneven (see Chapter 3).

Good immunoreactivity with Sp12 requires permeablization of larvae. The disruption of tissues attendant on fixation is not sufficient to allow Sp12 access to its epitope. Acetone or Triton X-100 extraction is essential to obtain the degree and clarity of labelling presented here.

Tissue culture supernatant, ascites fluid, and purified antibody of all three types show the same tissue specificity. Slight differences in non-specific binding of antibody (controlled for by NRH from an equivalent source) is occasionally evident, but specific binding is invariant. Protein A purified antibody, which presumably contains only IgG, has exactly the same specificity as crude supernatant or ascites. Dilution of antibody (ascites or supernatant) results in an alteration of Sp12's tissue specificity (Fig. 2). Supernatant diluted 1:1 or 1:10, and ascites diluted 1:250 or 1:500 labels all three cell types. At higher dilutions, supernatant at 1:100 or 1:500 and ascites at 1:1500 or 1:2500, skeletal rods and skeletogenic cells lose their immunoreactivity while blastocoelar cells and pigment cells retain it. The absolute levels of immunoreactivity are decreased, but this change in localization is unmistakable. At high antibody dilutions all cells lose their immunoreactivity, and appear the same as negative control specimens. Another method of antigen detection, western blot analysis (see Chapter 4), reveals no differences between blots when primary antibody (Sp12 ascites) is diluted out through the series 1:300, 1:600, 1:1200, 1:1800, and 1:2400.

Figure 2. Tissue specificity of Sp12 in 5d plutei. a) Spent tissue culture supernatant from Sp12 hybridomas, diluted 1:10 with filtered sea water (FSW). The skeletal rods, primary (skeletogenic) mesenchyme (arrow), and secondary mesenchyme (blastocoelar cells around the gut) (arrowhead) are all intensely labelled. b) The same supernatant diluted 1:100. Skeletal rod and primary mesenchyme labelling (arrow) is greatly diminished relative to the fusiform blastocoelar cell labelling (arrowhead). c) The same supernatant diluted 1:500. The skeletal rod and primary mesenchyme labelling is almost extinguished (arrow). Fusiform cells on the skeletal rods are blastocoelar cells (arrowhead). Pigment cells are poorly labelled in this batch of embryos, but other experiments show that they retain their labelling at high dilutions of Sp12. d) Embryo labelled with nonrelevant hybridoma (NRH) supernatant undiluted. The same batch of embryos is labelled at all dilutions. Scale bar is 20 μm .



DISCUSSION

The goal of these experiments was the production of mesenchyme specific monoclonal antibodies, yet the immunogen utilized was a mixture of crude pluteus and adult test extract. The use of crude extracts is based on two advantages: it is easy to produce immunogen and still get monospecificity during cloning of the hybridoma; and it allows for the discovery of completely uncharacterized antigens. The pluteus extract is an obvious source of larval antigen, but the rationale for inclusion of adult tissue may be less apparent. Test extract was utilized because cells within the adult test are known to express genes which are also expressed in larval primary mesenchyme (Drager *et al.*, 1989), thus test is a potentially abundant source of mesenchyme antigens, enriching the immunogen. This strategy appears to have been effective, and provides an explanation of the mesenchyme-test crossreactivity of Sp12 (detailed in Chapters 3 and 4).

The difficulties encountered in the initial attempts at monoclonal antibody production were likely related to two problems in the fusion procedures. First, the 50% concentration of PEG is probably toxic to the cells undergoing fusion (Kennett, 1979). There is a fine line between inducing sufficient membrane disruption to allow cell fusion without precipitating cell death. Second, the centrifugation of the cells while in the PEG may greatly increase fusion rates (Gefter *et al.*, 1977; Kennett, 1979). It makes intuitive sense that providing physical proximity and compression of adjacent cells should increase fusion rates. While these points have little bearing on the positive results of my research, they help explain the considerable time and effort spent obtaining negative results initially on this project.

One concern when producing monoclonal antibodies is establishment of their monoclonality. At issue here is whether Sp12 is in fact monoclonal. The technique of cloning by limiting dilution provides a reliable method of ensuring monoclonality, and this can be improved further by repeating the cloning (McKearn,

1980). Sp12 has remained absolutely consistent after each of the three clonings. An identical tissue specificity was found in all positive pots, and in the last two clonings over 95% of the growth positive clones were also positive for Sp12 secretion. The cell line is stable in culture over extended periods and cells frozen in liquid nitrogen retain their specificity and gross titre. Furthermore, isotyping shows a single antibody subtype is present in Sp12 supernatants. In light of these results the probability that the Sp12 cell line is polyclonal is extremely small. The possibility that a single cell line could be secreting two antibodies may be entertained, but this occurs very rarely, if at all. Within the limits of certainty, Sp12 is a monoclonal antibody.

Two antibody purification methods were utilized in this project, ion exchange and protein A columns. While ion exchange offers a rapid, one-step procedure (Boonekamp and Pomp, 1986), the eluant is contaminated with a number of extra proteins. This situation is not significantly altered by eluting with a shallow salt gradient. For experiments requiring precise and accurate quantification of antibody concentration this method is insufficient. Protein A provides very clean preparations of IgG antibody, as reported (Goding, 1986). However, this method has a greater chance of antibody loss through the purification, and a risk of antibody denaturation if buffers are not rapidly neutralized. In our hands, careful attention to these concerns allows good yields of IgG from protein A purified ascites. Fab production suffers from similar drawbacks, large drops in yield are inevitable with the number of purification, concentration, and proteolysis steps required.

Figure 2 illustrates an unusual property exhibited by Sp12. At low dilutions skeletogenic mesenchyme and skeletal rods are the most intensely labelled tissue in the embryo, while at high dilutions this tissue is almost completely negative for fluorescence. In comparison, blastocoelar cells appear to label more intensely as the antibody is diluted out. This paradoxical finding may be due to tissue specific

differences in Sp12 avidity for antigens on the two tissues (Berzofsky and Berkower, 1984). The differences in tissue specificity at different antibody concentrations are not seen in western blots incubated at decreasing concentrations of Sp12. Such blots show identical patterns and intensity of bands. Labelling of the Sp12 antigens appears to be unaffected by antibody concentration when they are not *in situ*. The epitope is hidden to some degree on all cells (hence the need for acetone or Triton X-100 pretreatment) and some additional peculiarity of the antigens' orientation on skeletogenic mesenchyme appears to preclude their labelling at low antibody concentrations. One note of caution must be inserted, Sp12 recognizes multiple antigens (see Chapter 4), and this crossreactivity may be due to slightly different epitopes (with different Sp12 affinities) rather than a single redundant epitope. In that case, differences in binding could be due to differences in affinity, not just avidity. Further investigation of this result is beyond the scope of this project, but whatever the explanation for this phenomenon, it provides a technique for examining blastocoelar cells without skeletogenic fluorescence interference.

More detailed investigations of the ontogeny of the Sp12 antigens and the tissues it labels were undertaken to clarify these aspects of its specificity.

CHAPTER 3 IN SITU ONTOGENY OF THE SP12 ANTIGENS

INTRODUCTION

Sp12 exhibits a unique tissue specificity which has not been described in the literature and was therefore chosen for more detailed investigation. From a developmental perspective, one of the first things requiring clarification was a complete ontogeny of the Sp12 antigen's expression. A description of the antigens' appearance *in situ*, through development, yields two important pieces of information: the temporal aspect of antigen expression may provide precise timing of the coding gene's expression; and the spatial localization of antigen expression may provide lineage information, and cell and tissue distributions. Combined, the timing and localization of antigens may provide clues as to their function in development.

Temporal expression of cell surface epitopes is generally a good indicator of the functional expression of genes coding for cell surface molecules. Regardless of whether the epitope is translational or a post-translational modification, its appearance on the cell surface has obvious implications for the cell's differentiation and for the function of the antigen.

Lineage analysis of cells and tissues is a potentially revealing temporal and spatial aspect of the *in situ* ontogeny. This pursuit has received generous attention in the sea urchin embryo. The regulative mode of development makes lineage analysis difficult, however, molecular probes including cloned nucleic acid sequences and monoclonal antibodies are revealing unexpected relationships. The ultimate goal of such analyses is an understanding of differentiation through examination of the early events underlying cell lineage allocation and gene regulation.

Localization of antigens (or other specifically labelled molecules), both histologically and ultrastructurally, provides further evidence of their function. Molecules involved in cell-environment interactions must be connected to the cell-environment interface. As this study is based on the hypothesis that cell surface molecules are important in metazoan morphogenesis, establishing that the Sp12 antigens are cell surface molecules was a prerequisite to further investigations. This can be done both histologically and ultrastructurally. In addition, ultrastructural identification of antigens can provide some information about their cellular metabolism.

The *in situ* ontogeny (temporal and spatial patterns of expression) and tissue localization of the Sp12 antigens not only initiate the characterization of these molecules, they may contribute evidence of the molecules' function.

MATERIALS AND METHODS

Embryonic and larval culture

Methods for obtaining gametes and raising embryos and larvae are outlined in Chapter 2.

Embryos for the prehatched ontogeny (stages before 24 hours PF) were fertilized in FSW with 1.0 mM aminotriazole (Sigma), which prevents hardening of the fertilization envelope. After washing twice with FSW embryos were allowed to develop in FSW for one hour, then fertilization membranes were removed by pushing the embryos through 50 μ m Nitex mesh (Showman and Foerder, 1979).

Merogones were produced by centrifugation of fresh, unfertilized eggs in a continuous 0 to 0.85 M sucrose gradient in artificial seawater (ASW, Jamarin U). Eggs were layered on top of the gradient and centrifuged (10,000g, 10 min). Two fractions resulted, a smaller, dense, nucleated merogone and a larger, less dense, lipid filled merogone. These fractions were removed, fixed and labelled as follows.

Other species were raised in an identical manner, or were obtained fixed from colleagues.

Ontogeny

Embryos and larvae were fixed as outlined in Chapter 2.

Indirect immunofluorescence was performed in three ml test tubes by incubating about 50 μ l of fixed, permeablized, embryos or larvae in 100 μ l of a 1:300 dilution of ascites fluid in FSW with 0.6% Triton X-100 (4°C, 16 hr). Unbound primary antibody was removed with three 1.5 ml washes in FSW, allowing the embryos or larvae to settle by gravity between washes. Fifty μ l of FITC-RAMIG, diluted 1:25 in FSW, was added to each tube and allowed to react (21°C, 30 min). Specimens were washed three times in FSW, mounted and viewed as described in Chapter 2. Photographs were taken with a Zeiss M35 camera on Kodak Tri-X film.

Adult tissues were fixed with 4% paraformaldehyde in FSW (21°C, 48 hr). They were washed briefly in DW and soaked in 20 volumes of 10% EDTA in DW for 96 hours (21°C), followed by fresh 10% EDTA in DW for another 36 hours. Carbon dioxide gas was removed from the tissue by a brief treatment under vacuum. Tissue pieces were dehydrated through alcohol to xylene, embedded in paraffin and sections were cut at 10 μ m thickness. Every two to three sections were mounted on separate slides, deparaffinized, and labelled using indirect immunofluorescence as described above, or stained with Masson's trichrome stain.

Immunogold localization

Immunogold specimens were fixed with freshly depolymerized 4% paraformaldehyde containing 0.1% glutaraldehyde (21°C, 1 hr) and dehydrated through a graded series of ethanol. Specimens were embedded in LR white which was polymerized under a nitrogen atmosphere (60°C, 24 hr). Thin sections were cut and collected on nickel grids, then processed for immunogold following DeMay (1983). Briefly, sections were blocked (21°C, 30 min) in BSA buffer (20 mM Tris-

HCl, 150 mM sodium chloride, 0.1% bovine serum albumin (BSA), pH 8.2) containing 1% normal goat serum by floating grids (with sections) on 100 μ l drops of the solution. Sections were then incubated in BSA buffer containing primary antibody ascites at a 1:50 dilution (21°C, 4 hr). After washing twice in BSA buffer (10 min on an orbital shaker) sections were incubated in Protein A-colloidal gold (10 nm diameter, BioCell) diluted 1:10 or 1:5 in BSA buffer (21°C, 1 hr). After washing, sections were fixed in 0.1% glutaraldehyde, dehydrated, contrasted with uranyl acetate and viewed on a Philips EM300 electron microscope.

Controls for immunoreactivity

All assays using Sp12 included a duplicate assay with NRH to control for nonspecific binding. Some procedures utilized Sp1, a monoclonal antibody specific to pigment cells in *S. purpuratus* (Gibson and Burke, 1985), as a positive control.

RESULTS

Ontogeny of the Sp12 antigens in situ

Indirect immunofluorescence of unfertilized eggs shows labelling of several hundred 1 μ m granules in the egg cortex (Fig. 3a). Optical sectioning of labelled eggs reveals that granules are closely spaced and evenly distributed around the periphery of the cell. The labelling outlines the granules, giving them a ringed appearance.

At one hour PF immunofluorescence encloses the embryo in a diffuse web and there is no evidence of the granules (Fig. 3b). Immunoreactivity appears extracellular, and occurs on both the zygote cell surface and in the space defined by the hyaline layer. Apparently Sp12 antigen released from dehiscing cortical granules is retained as a hyaline layer component.

Although hyaline layer immunoreactivity becomes more homogeneous in older embryos, it often has a bilaminar appearance with an outer ring of

immunofluorescence and an inner ring closely applied to the cell surface. This is apparent as early as the 16-cell stage (6 hr PF) (Fig. 3c), and is retained until at least the hatched blastula stage (24 hr PF) (Fig. 3d). Hyaline layer immunoreactivity is evident on fixed whole mount larvae until about the full gastrula stage, when it becomes patchy and fades.

Antigen is expressed on mesenchyme cell surfaces in mesenchyme blastulae (30 hr PF). Primary or skeletogenic mesenchyme start to express the Sp12 epitope two to three hours after they ingress into the blastocoel (Fig. 3e). However, strong immunoreactivity is not apparent until they have adopted their characteristic ring pattern around the vegetal pole of the embryo (Fig. 3f). These cells and the larval skeleton they secrete remain immunoreactive through the rest of embryonic and larval development.

Secondary mesenchyme (blastocoelar cells) start to exhibit Sp12 immunoreactivity after they are free of the archenteron epithelium. Cells at the archenteron tip, along the archenteron and blastoderm basal laminae and spanning the blastocoelar space between the two, express the epitope between half and full gastrula (44-48 hr PF) (Fig. 3g, 3h). However, Sp12 antigen expression is a highly variable event, and within a simultaneously timed batch of embryos one embryo may have no cells showing immunoreactivity at seven-eighths gastrula, while another at exactly the same stage may have five or six immunoreactive cells at the archenteron tip. At full gastrula the Sp12 epitope is expressed on 26 to 36 skeletogenic mesenchyme and on zero to six secondary mesenchyme cells (Fig. 3h).

By early prism (65 hrs PF) skeletogenic and blastocoelar mesenchyme are expressing the Sp12 epitope. After separating from the archenteron, blastocoelar cells are migratory on the blastodermal basal lamina and archenteron surface, and then become moored by their filopodial attachments. Chromogenic mesenchyme, released just at the onset of gastrulation (archenteron invagination), may start to

express the epitope by full gastrula, but pigment cells are not clearly identified and expressing the epitope until the prism stage. Muscle cells of the esophagus and coelomic cells never become immunoreactive.

In the prism (72 hr PF) skeletogenic mesenchyme, skeletal rods, and pigment cells continue to be immunoreactive (Fig. 3i). Blastocoelar cells (the prominent secondary mesenchyme derivative) are also clearly labelled, and include five to six cell bodies on the blastocoelar surface of the primitive gut, five or six on the skeletal rods, three or four suspended in the blastocoel by filopodia, and six to eight dispersed around the blastocoel closely apposed to the ectoderm.

Early plutei (96 hr PF) retain a similar pattern of skeletal and blastocoelar cell immunoreactivity. There is elaboration of the complexity and interconnections of these cells' processes (Fig. 3j). Immunoreactive pigment cells, readily identified by their characteristic pseudopodia with "footpads" at their ends, and by their location in the epidermis of the larva, are better defined and more numerous in these and older larvae, although their labelling remains variable in intensity.

Skeletogenic mesenchyme and skeletal rods continue to be prominently immunoreactive, and blastocoelar cells are clearly labelled in the pluteus (Fig. 3k). Blastocoelar cell processes form networks in developing arms, beside the ciliary band, around the gut, and along the skeletal rods. There is a slight increase in the number of immunoreactive blastocoelar cells between prisms (17 to 25 cells) and 5 day plutei (20 to 27 cells). There is a similar increase in number in later plutei as arms grow and are added (see Chapter 5 for a more detailed account of blastocoelar cells).

Older plutei (10 d PF) retain the same pattern of tissue localization as 5 day plutei, although cytoplasmic processes become attenuated and more complex in their interwoven patterns (Fig. 3l). Tissue specificity does not change in the oldest larvae examined (30 d PF). Adult urchins retain Sp12 antigens (see below) but the

ontogeny of the cells which produce adult immunoreactive material has not been determined. The complex events of adult rudiment formation and metamorphosis intervene between the larval stages described above and the adult localization described below.

Negative controls for the immunofluorescent ontogeny (Fig. 4) reveal the type and degree of non-specific fluorescence seen in probed embryos and larvae. In general, cytoplasmic and hyaline layer fluorescence are most common in embryos less than 48 hours old. Plutei commonly exhibit speckled fluorescence in cells of the stomach and intestine.

Sp12 antigen localization by immunofluorescence

Sp12 appears to be localized to granules in the cortex of unfertilized eggs. To determine if they are in fact cortical granules, merogones were produced and labelled. Merogones also have a cortical, granular, immunoreactive component. Granules are distributed evenly in the cortex of both merogone halves. The pattern of distribution in intact eggs and in merogones is identical, indicating that these granules are anchored in the cortex, and are not dislodged by strong centrifugal force.

The three mesenchymal derivatives labelled in the pluteus are skeletogenic cells (Fig. 5a,b), blastocoelar cells (Fig. 5c,d) and pigment cells (Fig. 5e,f). As shown by the light micrographs complementing the immunofluorescence micrographs in this figure, mesenchyme and its derivatives are difficult to visualize with light microscopy alone. Sp12 labelling highlights cells and their processes, and gives a detailed view of their morphology and location.

Blastocoelar cells are a secondary mesenchyme component which have extensive filopodial connections between the gut, spicules, and epidermis of the larva (Fig. 6a-d; see also Chapter 5). The morphology of the cells is characteristic, but the actual number of processes each cell has varies from two to several.

In plutei, blastocoelar cells appear syncytial and are clearly connected to each other and the primary mesenchyme by filopodia (Fig. 6a-d). There are large differences in the degree of pigment cell immunoreactivity between larvae, but even cells situated side by side in the same larva occasionally exhibit very different levels of immunoreactivity (Fig. 6e). The pattern of pigment cell labelling seen with Sp12 is identical to that seen with Sp1, a pigment cell specific monoclonal antibody (Fig. 6e,f)

Adult calcareous tissues also exhibit Sp12 immunoreactivity. Antigen is localized in the mesodermally derived dermis of the spines (Fig. 7a-c) and body wall (Fig. 7d-f). It occurs at the interface between the calcium carbonate skeletal matrix and the soft tissue of the skeleton (Fig. 7a,d,g). While there may be a syncytial cell membrane lining the calcium carbonate meshwork (stereom) making up the test, it is not possible to distinguish between extracellular matrix (ECM) and membranes in these sections. No cells are specifically labelled with Sp12, although brightly autofluorescent cells are present in both Sp12 and NRH probed sections of test (Fig. 7d,e). The Sp12 epitope could not be detected in tube feet, muscle, epidermis (Fig. 7g,i), gut, coelomocytes, or testis of the adult.

Ultrastructural localization

Immunogold labelling of unfertilized egg thin sections shows particles bound to the lamellar portion of cortical granules (Fig. 8a-c). About 80% of the granules are immunoreactive, and frequently one granule binds numerous gold particles while a similar adjacent granule binds none. Sections probed with NRH show low levels of nonspecific labelling similar to that of egg cytoplasm (Fig. 8a).

Gastrulae (48 hr PF) thin sections are not as intensely labelled as unfertilized eggs. Most gold particles are associated with mesenchyme in the blastocoel (Fig. 8d). Cell surfaces are clearly labelled, as are regions within the cytoplasm, specifically, Golgi-like structures and vesicles (Fig. 8e). The vesicles have antigen

associated with their perimeters (presumably the delimiting membrane) as well as with material inside the vesicles, apparently free of any membrane associations (Fig. 8d,e).

Prisms (72 hr PF) probed with immunogold show patterns of immunoreactivity similar to those of gastrulae. Gold particles are concentrated over mesenchyme, and are at background levels in other cell types. Again, labelling of vesicles and cytoplasm provides evidence of antigen production in these cells (Fig. 8f). In contrast, epidermal cells do not exhibit cytoplasmic labelling above background. There are occasional particles associated with the hyaline layer in prisms, although the degree of labelling is only slightly above background (Fig. 8g).

Interspecific crossreactivity

A variety of other echinoderms express the Sp12 epitope. Sp12 has the same tissue specificity in plutei of the green sea urchin *Strongylocentrotus droebachiensis* as in *S. purpuratus*. Skeletogenic and blastocoelar cells as well as one population of pigment cells (those containing red echinochrome pigment) all express the Sp12 epitope (Fig. 9a,b). Plutei of the sea urchin *Lytechinus pictus*, and the sand dollar *Dendraster excentricus* have the Sp12 epitope on their skeletogenic mesenchyme only (Fig. 9c,d). Brachiolaria larvae of the sea star *Pisaster ochraceous* and ophioplutei of the brittle star *Ophiopholis aculeata* do not exhibit any Sp12 immunoreactivity.

Figure 3. *In situ* ontogeny of Sp12 antigens. **a)** Unfertilized egg. This equatorial optical section shows the cortical distribution of the 1 μm granules labelled by Sp12 (arrowhead). The inset is a tangential optical section of the same specimen. **b)** One hour embryo. No evidence of cortical granules remains in this equatorial optical section, but a diffuse meshwork of antigen is apparent in the hyaline layer (arrowhead). Inset shows a tangential optical section of the same specimen. While fluorescence is quite diffuse, there is a vaguely fibrillar organization of the antigen. **c)** Sixteen cell stage, 6 hr. Hyaline layer labelling is still apparent, though the meshwork cannot be resolved. The antigen appears to have a bilaminar distribution in the hyaline layer - closely apposed to cells, and at the outer limit of the ECM (arrowhead). Cells of this distinctive cleavage stage can be discerned with mesomeres at the animal pole (top) and micromeres at the vegetal pole (bottom) of the embryo. **d)** Hatched blastula, 24 hr. Hyaline labelling is bilaminar, and although primary mesenchyme appear to have ingressed at the vegetal pole (arrow) no cellular fluorescence is apparent on these cells or in the vegetal plate. **e)** Early mesenchyme blastula, 30 hr. Cells have ingressed and started to migrate, but these mesenchyme are expressing little Sp12 antigen (arrow). Hyaline immunoreactivity is diminished relative to the previous stage. **f)** Late mesenchyme blastula, 38 hr. Mesenchyme cells have migrated up the blastoderm, and returned to form a ring around the vegetal pole. They are now expressing Sp12 antigen on their surfaces (arrow). Hyaline layer immunoreactivity has faded considerably. Scale bar is 20 μm .

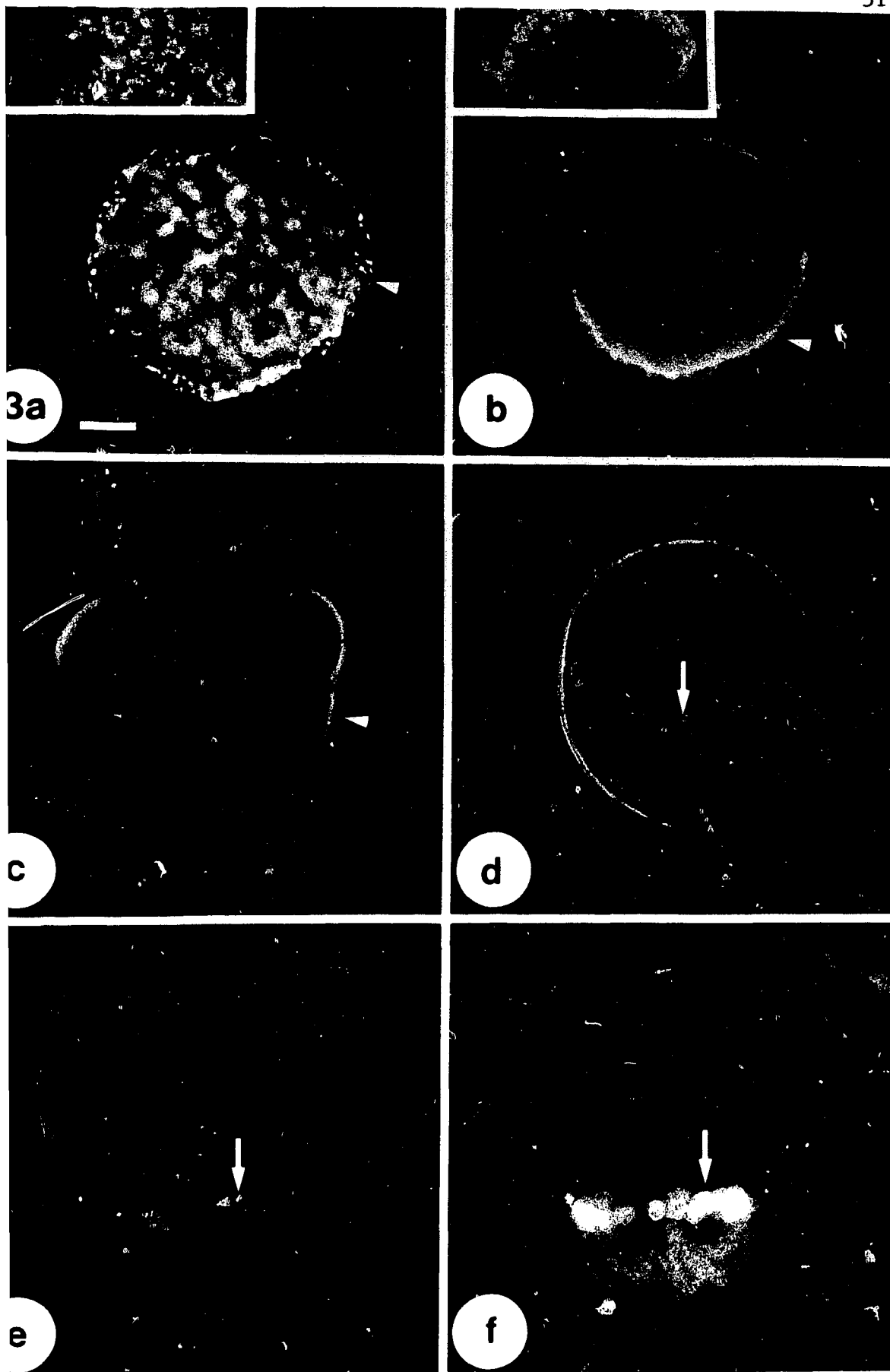


Figure 3 (continued). **g)** Half gastrula, 44 hr. Skeletogenic mesenchyme retain their intense immunoreactivity (at this concentration of antibody) through the rest of embryonic and larval development. While secondary mesenchyme are released from the archenteron by this stage, it is not clear whether the fusiform cell labelled by Sp12 (arrow) is a presumptive blastocoelar cell, or an errant skeletogenic cell. Hyaline layer labelling is no longer apparent. The diffuse cytoplasmic glow from the archenteron, vegetal plate, and animal plate are non-specific. **h)** Full gastrula, 48 hr. The ring of skeletogenic mesenchyme (s) with numerous fine filopodia is intensely labelled, and a few secondary mesenchyme cells at the animal pole (arrow) are expressing antigen. The number of secondary mesenchyme that are immunoreactive at this stage is highly variable (see text). **i)** Prism, 72 hr. Skeletogenic cells (s) are secreting the larval skeleton, and two secondary mesenchyme derivatives can be discerned. Multipolar blastocoelar cells (b) appear to connect distant portions of the embryo. Chromogenic mesenchyme, or pigment cells (c) start expressing Sp12 in the epidermis. **j)** Early pluteus, 96 hr. Skeletogenic and blastocoelar cells are clearly labelled. Pigment cell labelling is more variable, although one putative pigment cell (c) is apparent in this specimen. A tangential optical section of the embryo gives the clearest view of pigment cells. The blastocoelar cells are forming a network of fine filopodia throughout the blastocoel. **k)** Pluteus, 120 hr (5 d). Skeletogenic (s), blastocoelar (b), and pigment (c) cells are all labelled and visible in this lateral view. The blastocoelar meshwork of filopodia is extensive. **l)** Pluteus, 10 d. This ventral view reveals that the skeletogenic, blastocoelar, and pigment cell labelling is retained in advanced larval specimens. No change in tissue specificity is observed in any older larvae examined. Scale bars equal 20 μm in g-j, and 50 μm in k and l.

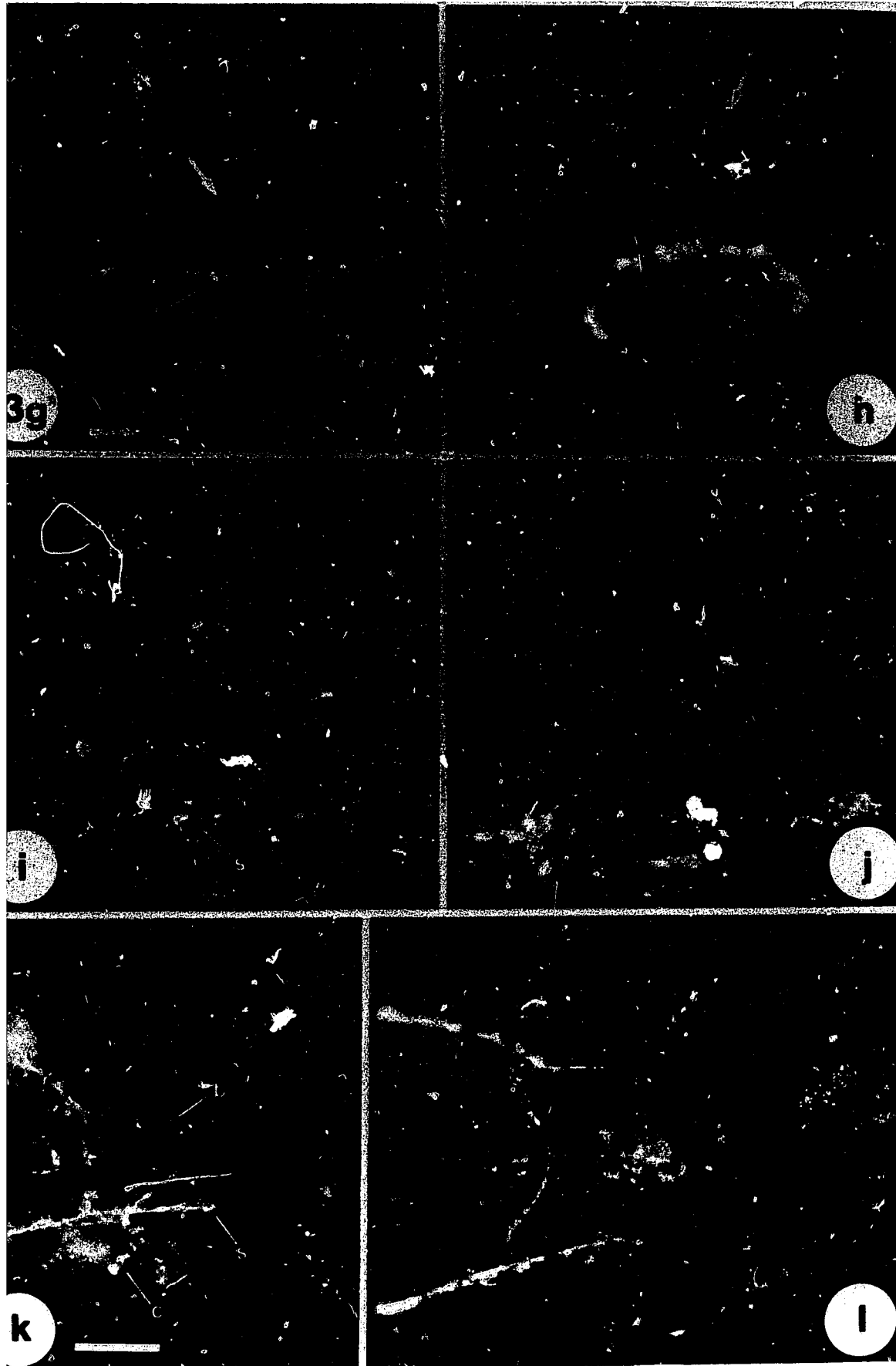


Figure 4. NRH labelling of sea urchin embryos and larvae. These negative controls indicate the amount of nonspecific labelling represented in the Sp12 labelling above. a) Unfertilized egg. The diffuse pale yellow cytoplasmic fluorescence seen in this and later stages is easily distinguished from the green fluoresceine fluorescence of Sp12 labelled structures. b) blastula, 20 hr. c) early mesenchyme blastula, 30 hr. d) Half gastrula, 44 hr. e) Full gastrula, 48 hr. f) Pluteus, 120 hr. The gut speckling shown here was variable, but was occasionally accompanied by ciliary band speckling. Scale bar is 20 μm in a-e, and 50 μm in f.

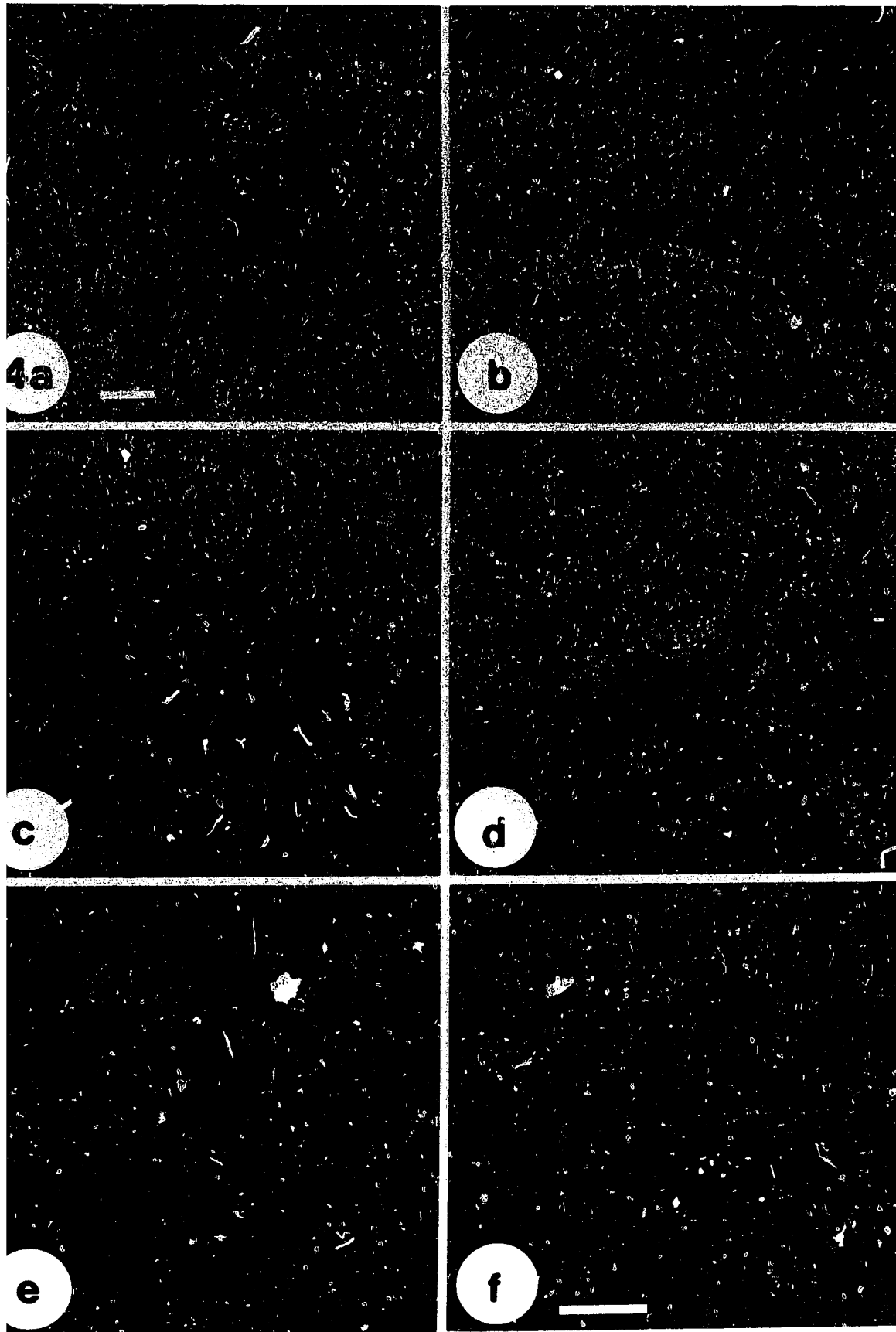


Figure 5. Mesenchyme derivatives labelled by Sp12. **a)** Skeletogenic mesenchyme. Both the cell body and the spicular envelope are immunoreactive at the 1:300 dilution of ascites fluid routinely used. **b)** Corresponding light micrograph. **c)** Blastocoelar cells. As indicated in Fig. 3 these cells form a complex network of interconnected cellular processes in the pluteus. **d)** It is difficult to discern these cells in whole mount light micrographs, yet Sp12 labelling provides a detailed image of them. **e)** Pigment cells in the larval epidermis. Characteristic footpads are outlined by Sp12 immunofluorescence. The intensity of pigment cell immunoreactivity is highly variable between embryos and between cells within one embryo. **f)** Corresponding light micrograph showing the remnants of pigment within these cells. Scale bar is 20 μm . Arrowheads indicate analogous cells.

Figure 6. High magnification views of Sp12 labelled cells in the pluteus. **a)** Ventral view of a post-oral arm. The skeletal rod and one skeletogenic cell (s) are flanked by blastocoelar cells (b) and processes which lie just under the epithelium on both sides of the skeletal rod. **b)** Lateral view of the "axilla" of the four arm pluteus. Blastocoelar cells connect the two skeletal rods, the ciliary band (cb), and each other through extensive filopodia and pseudopodia. **c)** Ventral view of the ciliary band between the two post-oral arms. Blastocoelar cells extend long processes (arrowheads) along the base of the ciliary band and attach to the skeleton laterally and the intestine posteriorly. **d)** Ventral view of the posterior stomach and *schietel*. Blastocoelar cells clasp the stomach (st) and send processes to the skeletal rods and epidermis from it. **e)** Pigment cells in the larval epidermis. Several blunt pseudopodia extend from the cell body of pigment cells, some of which have the footpads (arrows) seen in Fig. 5e. **f)** Pigment cells labelled with the monoclonal antibody Sp1. The distribution and morphology of the epidermal cells labelled by Sp12 and Sp1 are identical. Scale bar is 20 μm .

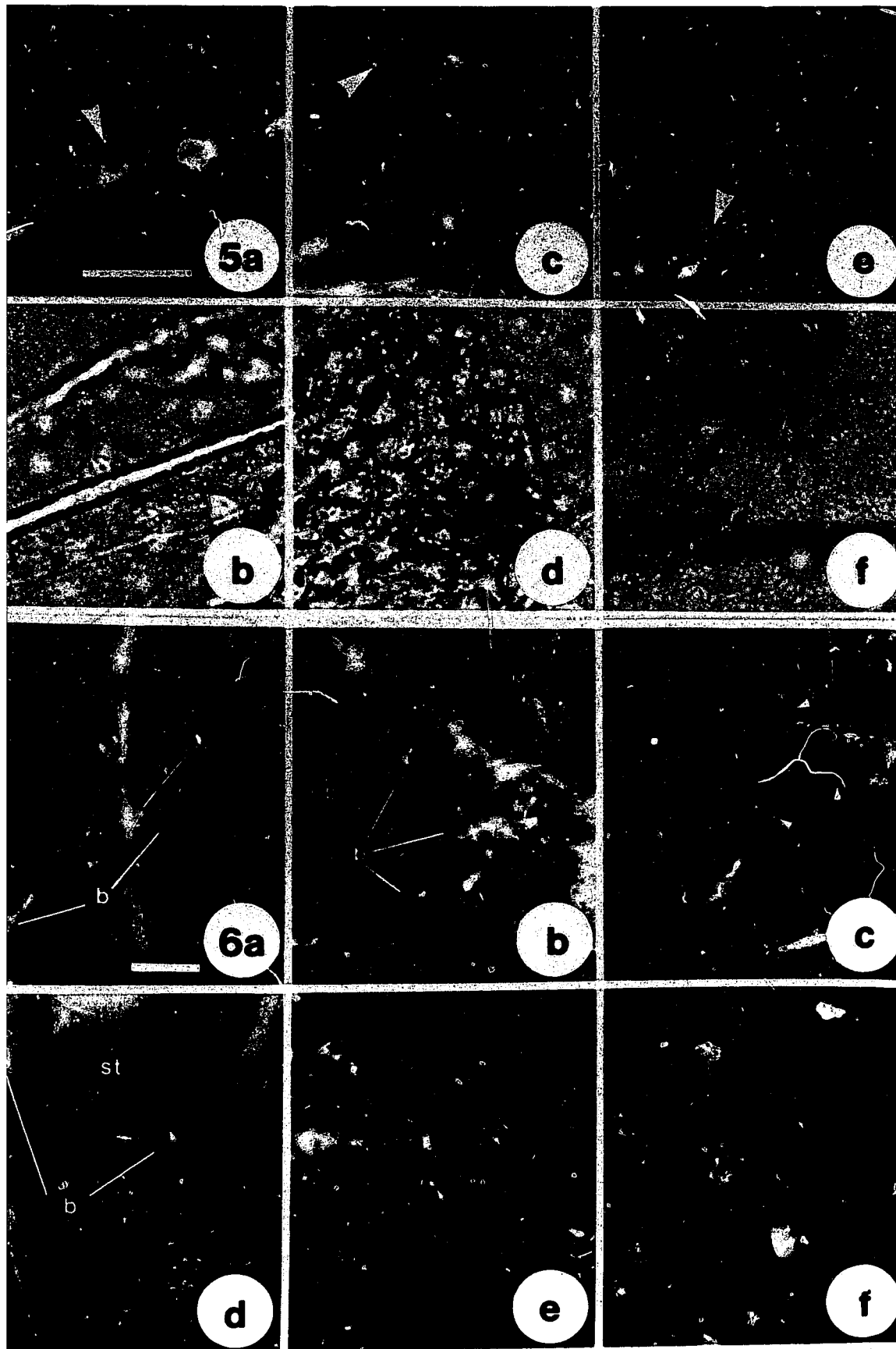


Figure 7. Adult tissue specificity. **a)** Sp12 labelled cross section of a spine. The fluorescence appears to define an ECM component that invests the calcium carbonate stereom. **b)** NRH labelling of an identical section. **c)** Light micrograph of the same section stained with Masson's trichrome stain. There is considerable disruption of the tissues due to processing. Despite this it is clear that Sp12 antigen is restricted to the dermal layer of the spine. The labelled tissue appears to be ECM, with discrete cellular elements embedded in it. **d)** Section through the test (t) with muscle fibres (m) adjacent. The material surrounding the mineral portion of the test is labelled. Muscle is autofluorescent, as are cells (arrow) within the matrix. **e)** Similar section labelled with NRH. The skeletal matrix is not fluorescent, while the muscle and cells (arrow) are. **f)** Similar section stained with Masson's trichrome. There appear to be many nuclei within the matrix. The large pigment cell (arrow) may be the autofluorescent cell type. **g)** Portion of test and spine with spine muscle intervening. Again, only the matrix appears to be labelled. **h)** Similar section labelled with NRH. No brightly autofluorescent cells are visible, but the muscle autofluorescence is confirmed (arrowheads). **i)** Low power tangential section of the urchin surface. Primary spines (ps) and smaller calcified structures which may be secondary spines (ss) and pedicellaria (pd) are labelled. Tube feet and the muscle and epidermis of the other structures are not labelled. **j)** Light micrograph corresponding to i) stained with Masson's trichrome. Scale bars equal 20 μm in a - f, 50 μm in g and h, and 100 μm in i and j.

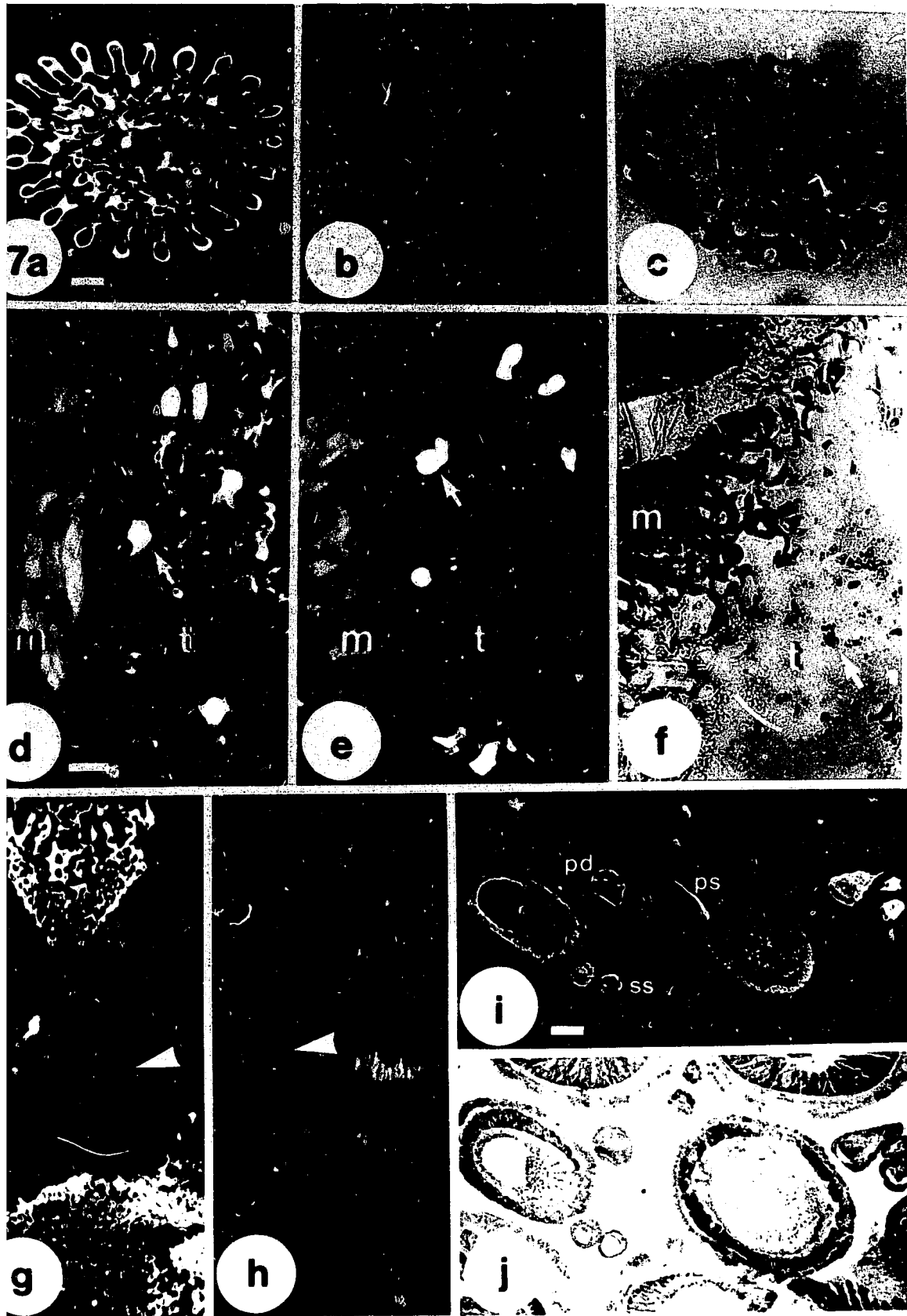


Figure 8. Immunogold localization of Sp12 antigen. **a)** Cross section of an unfertilized egg. Black dots are 10 nm colloidal gold particles. Antigen is clearly restricted to the lamellar portion of the cortical granules. Occasional particles in the cytoplasm are indicative of the level of NRH labelling of these sections. **b)** There is heterogeneity in the labelling of these granules, densely labelled granules are occasionally situated beside completely unlabelled granules (arrowhead). Less than 20% of granules are unlabelled. **c)** A densely labelled cortical granule. In this case the antigen appears to be localized to the electron dense lamellae of the granule. **d)** Blastocoelar cell in a 48 hr embryo. Antigen is localized to the cell surface of the whole cell, as well as to the limiting membrane of clear vesicles. Cell processes within the blastocoel are also labelled (arrows). Epidermal cells and NRH probed sections exhibit a low degree of labelling. **e)** High magnification view of a 48 hr blastocoelar cell. Antigen is localized to Golgi-like structures (G) and to the flocculated material within vesicles (v), as well as with the cell membrane (arrowhead) (n = nucleus). **f)** Blastocoelar cell in a 72 hr embryo. Antigen is still localized to the cell surface, as well as to vesicles within the cytoplasm (v). Processes within the blastocoel also retain their labelling. **g)** Epidermal cell and hyaline layer adjacent to the cell in f). A small amount of label is associated with the hyaline layer and the apical surface of the epidermal cell (arrowheads). The few grains within the cell's cytoplasm are equivalent to the level of NRH binding in these sections. Endodermal cells were not labelled with Sp12 at all. Scale bars are 400 nm in a, d, f, g, and 200 nm in b, c, e.

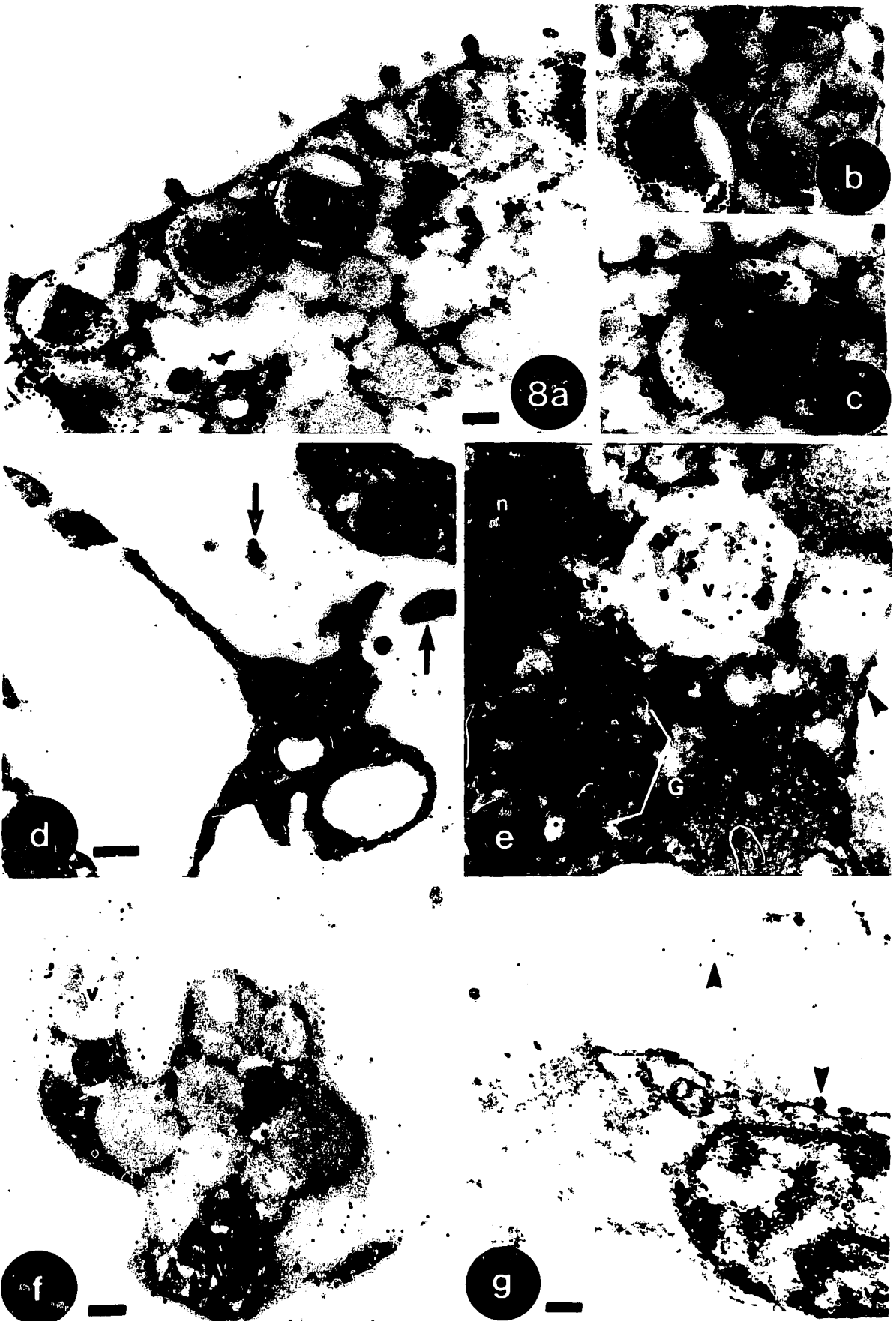
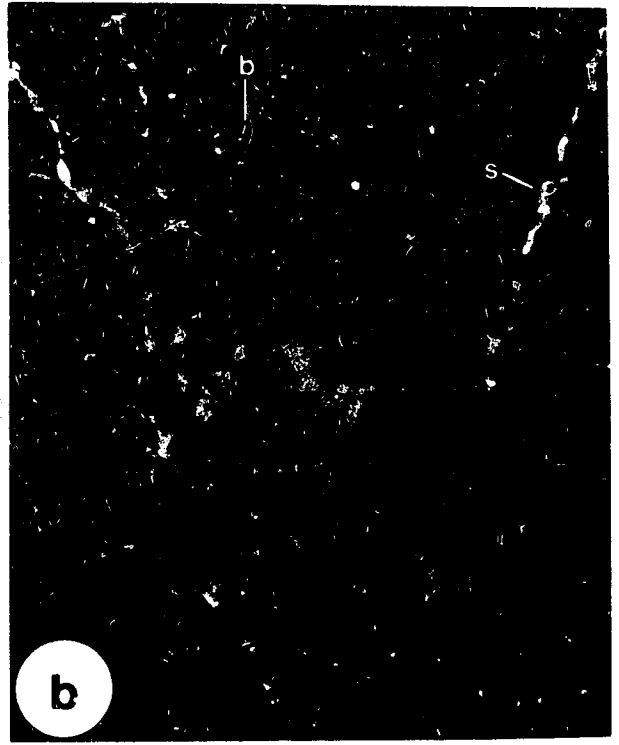


Figure 9. Tissue specificity of Sp12 in other species of echinoids. **a)** Ten day *Strongylocentrotus purpuratus* pluteus. As previously described, skeletogenic (s), blastocoelar (b), and chromogenic (not clearly seen here) cells are labelled. **b)** Five day *S. droebachiensis* pluteus. Although there is high background autofluorescence, there is clearly skeletogenic (s) and blastocoelar (b) cell labelling in this micrograph. Other experiments showed one of the two pigment cell populations is also immunoreactive. **c)** *Lytechinus pictus* four day pluteus. Only the skeletogenic cells (s) and skeletal rods are immunoreactive. Blastocoelar cells are not labelled (arrow). **d)** Five day pluteus of *Dendraster excentricus*. Again, skeletogenic cells (s) and skeletal rods are immunoreactive while blastocoelar cells are not (arrow). Scale bar is 50 μm .



DISCUSSION

In the course of examining the immunofluorescent *in situ* ontogeny, a degree of variability in the labelling became apparent. While this variability has been controlled with modifications to the assay procedure, a base level remains. No mention is ever made of such variability in published descriptions of anti-sea urchin embryo monoclonal antibodies, although I suspect this does not mean it never occurs with them! Immunolabelling is a labile process, and subtle changes in parameters may dramatically affect results. Such parameters include genetic variability between batches of embryos, variations in fixation and length of storage of specimens, the number of embryos used in the assay, the batch and age of ascites used, the timing of incubation, and the extent of washing. Another factor that is difficult to control is the subcellular localization of the epitope on the cell surface. It appears that the Sp12 epitope is poorly accessible in fixed embryos unless cell membrane lipids are partially removed with nonionic detergents or acetone. This indicates that the epitope may be closely apposed to the plasma membrane, and if crosslinked into place (by fixation) requires dissolution of the membrane to allow access by the antibody. Clearly, an epitope which requires special access could be subject to more variability in its labelling.

Despite these caveats, handled correctly Sp12 is a reliable and impressive antibody for histological and ultrastructural localization of these antigens.

Cortical granule and hyaline immunoreactivity

Unfertilized eggs labelled with Sp12 and viewed by indirect immunofluorescence had specifically labelled granules in their cortex. The merogone experiments confirm that these granules are anchored in the cortex. Furthermore, the immunogold labelling localizes Sp12 antigen to the lamellar portion of ultrastructurally identified cortical granules (Afzelius, 1956). This portion of the cortical granule is thought to contain a heterogeneous mix of

glycoproteins while the homogeneous region contains hyalin (Hylander and Summers, 1982). The localization of mesenchyme cell surface molecules to this maternally produced ECM compartment is unexpected, however, it has been documented with two other mesenchyme cell surface monoclonal antibodies. Anstrom *et al.* (1988) found about 20% of cortical granules were labelled with the monoclonal antibodies B2C2 and 1G8. Both of these antibodies recognize a post-translational modification on the primary mesenchyme-specific glycoprotein msp130. In contrast, Decker *et al.* (1988) labelled all cortical granules with 1G8 and another msp130 antibody, 1223. They proposed that poor fixation could be the cause of differential cortical granule labelling. The fixation schedule employed in this study is similar to that of Decker *et al.* While an artifactual cause of the 80% labelling observed cannot be ruled out, these data confirm Anstrom's findings, and indicate that the cortical granules are not an homogeneous population, but are antigenically diverse.

After fertilization, the granules are replaced with an initially felt-like mat of immunoreactivity, followed by a more homogeneous fluorescence in the hyaline layer. Hyaline layer immunoreactivity fades with further development and is no longer apparent after full gastrula. Immunogold labelling of 48 hr and 72 hr embryos supports this finding. Epidermal cells do not have antigen in their cytoplasm, although vestiges of antigen are still localized to the cell surface and in the hyaline layer. The hyaline layer antigens appear to be produced during oogenesis and packaged into the cortical granules. They are released into the hyaline layer at fertilization, but are not replaced during subsequent rejuvenation of this ECM. This is consistent with current views of hyaline layer secretion (Kane, 1973; McClay and Fink, 1982). The major protein in this ECM, hyalin, is initially exocytosed from cortical granules at fertilization, then secreted again at late blastula (Kane, 1973) or before gastrulation (McClay and Fink, 1982). Although the timing

of this rejuvenation of the hyaline layer is not exact, it occurs in the interval during which Sp12 labelling of the hyaline layer decreases.

The apparent bilaminar labelling of the hyaline layer seen in immunofluorescence micrographs, as well as in the immunogold micrographs indicates there may be two populations of Sp12 antigens: one tightly associated with the plasma membrane, and the other associated with a more easily extracted extracellular matrix. The stratified nature of the hyaline layer has been well documented (Lundgren, 1973; Spiegel and Spiegel, 1979; Cameron and Holland, 1985; Spiegel *et al.*, 1989). In addition, some components of the hyaline layer are known to be in intimate contact with the underlying cells (Hall and Vaquier, 1982). The layering of this ECM, and differential affinity of components to blastodermal cell membranes, could allow the segregation of Sp12 antigens to different levels.

The sharing of antigens (or at least epitopes) by the hyaline layer and mesenchyme cell surfaces is odd. It is possible that the ECM or peripheral membrane components of the apical and basal blastoderm require common components, the basal (blastocoelar) component being secreted or presented by mesenchyme. As both ECMs are involved in morphogenesis (see Chapter 1), this could provide the basis for the sharing of molecules. While this hypothesis is based on shared structural features, another possibility is that these antigens are shared for metabolic reasons. Farach *et al.* (1987) postulate that msp130 is involved in mediating calcium-uptake during spiculogenesis by primary mesenchyme cells, and note that some events during and immediately following fertilization are also mediated by calcium (*e.g.* membrane fusions, and hyalin precipitation). Msp130 may be involved in calcium regulation early in development as well as during spiculogenesis.

Too little is known about the function of the Sp12 antigens to indicate if either of these hypotheses apply to these molecules.

Mesenchyme specificity of Sp12

The second phase of immunoreactivity starts at mesenchyme blastula when skeletogenic mesenchyme start to express antigen on their surfaces. This and the later secondary mesenchyme expression occur after the cells have released from the blastoderm or archenteron tip. Unlike the other sea urchin mesenchyme antigens identified to date - msp130 (Carson *et al.*, 1985; Leaf *et al.*, 1987; Farach *et al.*, 1987, Anstrom *et al.*, 1987; Shimizu *et al.*, 1988), Meso1 (Wessel and McClay, 1985; McClay *et al.*, 1987), and the Sp1 antigen (Gibson and Burke, 1985) - which are expressed prior to or immediately at ingression, maximal Sp12 antigen expression is not apparent until well after the cells have started their migratory phase. In some cases it appears that the cells are fixed in place before Sp12 expression is maximal. A clear exception to this is the pigment cells which do not become fully immunoreactive until they are in their final site, but are never fixed in place. The timing of Sp12 antigen expression indicates that they are not involved in ingression from the blastoderm or archenteron, or the initial events of migration. However, this temporal pattern of expression is not inconsistent with their playing a role in cell recognition or the establishment and maintenance of spatial relationships between cells.

Another aspect of Sp12 labelling that is unique is the pan-mesenchyme specificity of the antibody. This localization distinguishes the Sp12 epitope from all other mesenchyme antibodies described to date. Most of these other antibodies are restricted to msp130 on skeletogenic mesenchyme, but Sp1 labels chromogenic cells (Gibson and Burke, 1985), Endo16 labels blastocoelar cells (Nocente-McGrath *et al.*, 1989), and Meso1 is expressed on skeletogenic and blastocoelar cells. The Meso1 labelling has been poorly documented (Wessel and McClay, 1985; McClay *et*

al., 1987), but is reported to distribute to the blastocoelar ECM in older larvae, a trait never displayed by Sp12.

While Sp12's temporal and spatial patterns of expression are unique and indicate a degree of molecular homology between these mesenchyme, they eliminate Sp12's use as a lineage marker in early development. The epitope is expressed too late to indicate precursor cells, and labels too many cell types to allow the singling out of cell lines. It is not possible to distinguish between the subsets of mesenchyme using Sp12 alone - cell morphology or other, late differentiating characteristics must be employed to accomplish this. The relationships of these cells and the implications of sharing the Sp12 epitope are presented in a detailed discussion in Chapter 5.

Ultrastructural localization

Immunogold localization of Sp12 antigens in the embryo reveals several salient points. The epitope is apparent in the Golgi apparatus, as well as in cytoplasmic vesicles and on the plasma membrane. From this pattern of localization it is clear that mesenchyme are actively producing Sp12 antigen through development. As indicated above, epidermal cells do not appear to produce Sp12 antigen, nor do endodermal cells. The localization of antigen to the limiting membrane of vesicles, as well as to the flocculated contents of vesicles indicates these antigens may have more than one degree of membrane affinity. Within the blastocoel, antibody is occasionally associated with wispy material adjacent to cellular processes. This localization of antigen is consistent with the vesicular labelling observed, and while the possibility of artifact cannot be ruled out, Sp12 antigens may segregate to both the mesenchyme cell membrane, and to the blastocoelar ECM. If they do localize to blastocoelar ECM, it is an ECM that remains closely applied to the cells. Neither the immunofluorescence nor

immunogold labelling indicate antigen is present anywhere but in the immediate vicinity of mesenchyme cells.

Adult tissue immunoreactivity

The portion of the adult body that is Sp12 immunoreactive is analogous to the larval blastocoelar space. It is situated between the ectodermal epithelium and the coelomic epithelium, and contains the skeleton and connective tissue (Hyman, 1955). During formation of the adult rudiment there is a migration of mesenchyme from the larval blastocoel into the presumptive adult body wall (MacBride, 1903). These cells may contribute to the formation of the test and secrete Sp12 antigens in the adult as they did in the larva. The migratory mesenchyme which contribute to adult structures have also been observed by Burke (1980b). The blastocoelar mesenchyme labelled by Sp12 do not appear to be migratory, although the above authors were examining different species, and more importantly, at a much later time in larval development. More detailed examinations of this larval - adult transition could help explain the origins of adult mesodermal tissues.

While Sp12 immunoreactive material appears to encase the larval skeleton and the adult test stereom in a similar manner their homology has not been directly compared. Biochemically, the organic matrix of the spicule and echinoid test are composed of mixtures of glycoproteins rich in hydrophilic amino acids (Benson *et al.*, 1986; Swift *et al.*, 1986; Weiner, 1985). The larval skeleton is sheathed in the plasma membrane of skeletogenic mesenchyme cells (Decker *et al.*, 1987), and the calcium carbonate is secreted onto concentric lamellae of organic matrix (Benson *et al.*, 1983). Immunogold and immunofluorescent labelling of spicules shows antigen associated with skeletogenic cells and the surface of the spicule, consistent with a cell membrane localization, although closely apposed ECM could also be involved.

Markel and Roser (1983) describe adult test structure in fine detail. Apposition of sclerocyte (or calcoblast - the calcium carbonate depositing cell) cell

bodies and numerous processes against trabeculae form membranous layers around them. In addition, an electron-dense ECM, the trabecle coat, invests the trabeculae. The cells of the stroma (the space between calcite trabeculae) in spines have been described in some detail. Heatfield (1971) and Heatfield and Travis (1975a; 1975b) provide detailed descriptions of them and their roles in regeneration of broken spines. Cells are not specifically labelled by Sp12 in test, making it unlikely that the trabecular immunofluorescence is due to sclerocyte cell membrane antigens. It appears that larval skeleton is labelled via the skeletogenic mesenchyme cell membrane while adult test is labelled via the trabecle coat ECM.

Larval and adult expression of these antigens may indicate a conserved functional relationship or may represent a lineage restricted marker for mesenchyme derived ECM. The conservation of gene products over the larval - adult transition has been examined for primary mesenchyme specific gene sequences (Parks *et al.*, 1988; Richardson *et al.*, 1989; Drager *et al.*, 1989). Drager *et al.* identify three primary mesenchyme specific mRNAs that are expressed in adult calcareous tissues. Two of these are present in calcoblasts (sclerocytes) involved in the regeneration of broken spines. The lack of adult cellular staining with Sp12 may be similar to their observation that calcoblasts in non-regenerating tissues do not express these PMC genes. Sp12 labelling has not been examined in regenerating systems, but Drager *et al.*'s results allow the possibility that cellular labelling could occur in that situation.

Adult mRNA from nonreproductive tissues hybridize to about 10% of gastrula mRNA (Galau *et al.*, 1976). Reciprocal hybridizations indicate that a significant proportion of the active gene set in adult tissues is in this shared mRNA. While this implies that most shared mRNA's are derived from house-keeping genes (Galau *et al.*, 1976), the Sp12 antigens have other qualities to distinguish them. In both larvae and adult test, antigens are cell surface or ECM molecules, and in both

cases are associated with calcified tissues. These common characteristics argue for a more specific structural role for these antigens.

Species specificity of Sp12

The conservation evident across the larval-adult transition is also evident in the range of species expressing the Sp12 epitope. Sp12 has been used by other researchers working on a variety of species (Wray and McClay, 1988; 1989). They confirm the *S. purpuratus*, *L. pictus*, and *D. excentricus* labelling. In addition, they report that Sp12 labels only skeletogenic cells in embryos of the urchins *L. variegatus*, *Arbacia punctulata*, the sand dollar *Mellita quinquesperforata*, and the primitive cidaroid *Eucidaris tribuloides*. This epitope is present in larvae of widely separated groups of echinoids, although it has not been identified in any of the other echinoderm families. The evolutionary importance of this occurrence is addressed by others (Wray and McClay, 1988; 1989), however, it is apparent that the Sp12 epitope has been strongly conserved. The pattern of conservation is interesting in that the only congeneric species examined (*S. droebachiensis*) retains the pan-mesenchyme specificity of *S. purpuratus*, while all other species express Sp12 antigens on their skeletogenic mesenchyme only. For the present, I can only speculate that the conservation of the Sp12 epitope could reflect some advantageous functional attribute.

The tissue specificity and localization of the Sp12 antigens uncovered some unexpected complexities in their expression. The presence of mesenchyme antigens in the cortical granules and hyaline layer is odd, but may reflect a common molecular requirement in these apparently diverse locations. The images of larval mesenchyme yielded by this pursuit are striking, and they confirm the cell surface location and mesenchyme secretion of these antigens. A clue as to the potential role of the Sp12 antigens is provided by the common behavioral attributes of the

labelled cell types. Further clarification of the larval-adult and the cross-specific sharing of Sp12 antigens may reveal much about their function in development. Visual images are one method of characterizing Sp12's specificity, but molecular questions, such as the M_r and chemical nature of the Sp12 antigens, require biochemical techniques. Chapter 4 describes these biochemical investigations.

CHAPTER 4 BIOCHEMICAL ONTOGENY AND ANALYSIS

INTRODUCTION

One of the powerful attributes of monoclonal antibodies is their capacity to label and identify a single molecular determinant in a heterogeneous mixture of many molecules. This allows a precise molecular dissection of biological events, including those involved in an organism's development. As outlined in Chapter 1, an understanding of developmental mechanisms requires detailed information on the molecular components involved. Many such components have been identified in the ECM of sea urchin embryos, and there is a growing number of cell surface molecules which have been described (reviewed in Chapter 1). Despite these recent discoveries, it is probable that these complex events rely on numerous interacting molecules, and it is unlikely that all of them are now known. Biochemical identification and classification of the relevant cell surface molecules is an essential prerequisite to any comprehensive model of morphogenesis in development.

The previous chapter provided *in situ* documentation of the Sp12 antigens' temporal and spatial expression. In this chapter western blot analysis identifies the molecules (based on M_r) labelled by the antibody, and allows temporal and spatial recognition of specific antigens through development. Temporal analysis (done by harvesting sequential samples from a developing batch of embryos, and running them on SDS-PAGE), and spatial analysis (accomplished by separating tissues of the developing embryo/larva using a number of different techniques, then analyzing these fractions with SDS-PAGE) refine the spatial and temporal expression patterns of the antigens first identified in Chapter 3. In addition, biochemical analysis provides details of these antigens' chemical nature, increasing the catalog of information regarding these molecules. Such a catalog is useful when trying to

evaluate the function of antigens, and provides distinguishing features which allow conceptual organization of these cell surface molecules.

MATERIALS AND METHODS

Polyacrylamide gel electrophoresis

To examine the apparent molecular weight of the antigens recognized by Sp12, samples of a number of developmental stages were run on polyacrylamide gel electrophoresis (PAGE), then western blotted and probed with antibodies. Sample buffer was composed of 3.9 ml DW, 1 ml 0.5 M Tris-HCl, pH 6.8, 0.8 ml glycerol, 1.6 ml 10% sodium dodecyl sulphate (SDS), 0.4 ml 2-mercaptoethanol, and 0.2 ml 0.05% bromophenol blue. Increased concentrations of sample buffer had less water and proportionally more of the other components. The 2-mercaptoethanol was omitted in non-reducing sample buffer. Samples were pelleted, resuspended in four to six volumes of sample buffer, immersed in a boiling water bath for 1.5 minutes, vortexed for 30 seconds, and either used immediately or stored at -20°C. All samples were centrifuged at 15,000g for 5 minutes before loading the supernatant onto gels.

PAGE was conducted using the Laemmli (1970) system with 7.5% (Cold Spring Harbour formula: 7.5% acrylamide, 0.2% bisacrylamide), 0.75 mm slab gels on a Hoeffer minigel apparatus. Gels were run at a constant current of 20 milliamps with ice packed against the vertical buffer chamber to provide cooling. Molecular weight standards used included the MW-SDS-200 kit (Sigma), 17kD-130kD Coomassie-conjugated standards (Biorad), and the MW-SDS-Blue kit (Sigma). Gels were stained with Coomassie blue dye, silver stain, or utilized in western blots.

Western blots

The basic procedure was taken from Burnette (1981). Gels were removed from the electrophoresis plates and assembled into sandwiches composed of: a perforated plastic plate, a scotchbrite pad, a Whatman number 4 filter paper, the gel, 0.45 μ m nitrocellulose membrane (Biorad), filter paper, scotchbrite pad, and perforated plastic plate. Some blots were done on a Hoeffer MiniMight western blotting apparatus. During assembly the sandwiches were saturated in degassed transfer buffer: 25 mM Tris-base, 192 mM glycine, and 20% methanol, pH 7.2. Sandwiches were oriented with the nitrocellulose on the anode side of the gel and 2.5 hour transfers were carried out, with cooling, at 10 volts per cm separating the charged plates.

Nitrocellulose was either stained immediately with amido black, or was incubated (37°C, 1 hr) in blocking buffer: 20 mM Tris-HCl, 150 mM sodium chloride, 0.1% BSA, pH 8.2, with 5% skim milk powder. Primary and secondary antibodies were diluted in this blocking buffer, and washes were done using the same buffer without the milk powder. After blocking, blots were incubated with primary antibody ascites (Sp12 or NRH) diluted 1:300 (21°C, 4 hr). They were then washed five times, 10 minutes each, in BSA buffer, with the middle three washes containing 0.1% Tween-20. The blots were incubated in the secondary antibody (RAMIG conjugated to HRP), diluted 1:400 (4°C, 12 hr). Occasionally incubation parameters for the primary and secondary antibodies were reversed. After washing five times as above, the blots were developed in 24 ml of chloronaphthol developer: 12 mg chloronaphthol (Sigma) in four ml methanol; 20 ml of 50 mM Tris-base, 200 mM sodium chloride, pH 7.4; and 82 μ l of 3% hydrogen peroxide. The reaction was stopped by washing with distilled water. For increased sensitivity blots were developed with diaminobenzidine (DAB).

Some blots were probed with concanavalin A conjugated to HRP (diluted 1:200 in BSA blocking buffer). This procedure was identical to the antibody procedures except no secondary antibody was required, and incubations were longer (4°C, 16 hr). Blots were also stained with periodic acid/Schiff's reagent (PAS). For this procedure blots were washed in PBS (3 hr), incubated in 0.6% sodium metaperiodate with 0.3% nitric acid in DW (21°C, 5 min), followed by Schiff's reagent (21°C, 10 min). They were then washed in several changes of 0.5% sodium metabisulphite in DW (21°C, 5 min each), rinsed in DW, and dried.

Immunoprecipitations

Immunoprecipitations were carried out as described in Adelson (1985). Embryos or larvae were pelleted by centrifugation (400g, 5 min) and the pellet resuspended in four volumes of immunoprecipitation assay buffer (IPAB): 10 mM Tris-base, 150 mM sodium chloride, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 25 mM EDTA, pH 7.2; plus a protease inhibitor cocktail, 0.015 mM pepstatin-A, 0.05 mM epoxysuccinylleucylamido(4-guanidino)butane (E-64), 0.1 mM N-p-tosyl-L-lysine chloromethyl ketone (TLCK), 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM iodoacetamide, and 1 mM ethyleneglutaminetetracetic acid (EGTA). The suspension was held at 21°C for 30 minutes with occasional vortexing to solubilize antigens before storing at -20°C. Adult test was treated identically except that it was first cleaned of any soft tissue, then ground in a mortar and pestle with approximately two volumes of IPAB.

Pansorbin *Staphylococcus aureus* cells (Calbiochem), were activated by centrifuging the cells (15,000g, 1 min), and washing the pellet in 10 volumes of activation buffer (AB): 20 mM Tris-HCl, 150 mM sodium chloride, 1 mM EDTA, pH 8.3; in the following sequence. The pellet was first suspended in AB with 0.5% Triton X-100 (21°C, 20 min), centrifuged (15,000g, 1 min), then resuspended in AB

with 0.05% Triton X-100 (21 °C, 20 min), centrifuged, and resuspended in AB with 0.05% Triton X-100 and 1 mg per ml BSA (21 °C, 20 min). The final pellet was resuspended in nine volumes of IPAB to make a 10% suspension.

The samples were thawed and centrifuged (20,000g, 1 hr). One ml of supernatant was precleared by adding 10 μ l of 10 mg/ml RAMIG, incubating (0 °C, 1 hr), then adding 200 μ l of a 10% suspension of activated Pansorbin and incubating (0 °C, 30 min) with occasional vortexing. These suspensions were centrifuged (15,000g, 5 min) and the supernatant was incubated with 30 μ l of Sp 12 ascites (approximate antibody concentration of 2.5 mg/ml) for 4 hours at 4 °C. Two hundred μ l of 10% Pansorbin was reacted with 40 μ l of RAMIG (0 °C, 2 hr), washed with IPAB, added to the one ml of Sp 12-antigen solution, and incubated (4 °C, 1 hr) with occasional vortexing.

Samples were centrifuged (15,000g, 5 min) and pellets were washed in IPAB, then once each in: R1 - 2 M sodium chloride, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM Tris-base, pH 7.2; R2 - 1% Triton X-100 in PBS, pH 7.2; and R3 - 50 mM sodium chloride, 0.5% Triton X-100, 10 mM Tris-base, pH 7.2. Final pellets were resuspended in four volumes of 1.25X sample buffer and immersed in a boiling water bath for 1.5 minutes, then vortexed for 30 seconds. Samples were centrifuged (15,000g, 5 min), and the supernatants were run on a gel immediately or stored at -20 °C.

Proteolysis and epitope determination

To ensure that proteolysis was not a factor in producing the pattern observed on western blots, samples were held under various conditions before immunoprecipitation. Early plutei (96 hr PF) were suspended in four volumes of 1% AB and held: at 0 °C, 30 minutes, with the protease inhibitor cocktail described above; at 21 °C, 30 minutes, with and without the protease inhibitor cocktail; and at 4 °C, 24 hours, with and without the protease inhibitor cocktail. Following these

treatments samples were immunoprecipitated and western blotted as outlined above.

Determination of Sp12's epitope required a large amount of antigen to subject to enzymatic digestion and chemical degradation. Crude extracts of the adult test provided such a source. Test was cleaned of soft tissue with a wire brush and pulverized in a mortar and pestle in PBS or 1 M glycine with 1% Triton X-100, and 0.05% SDS. This solution was centrifuged (25,000g, 1 hr), and the supernatant was utilized immediately or dialyzed against DW, lyophilized and stored frozen (-20°C).

Initially test extract was treated in solution with: protease VII, protease XIV, ribonuclease A, hyaluronidase, chitinase, and proteinase K, (all from Sigma), at 50 µg/ml in PBS; and with 50 mM sodium meta-periodate in DW, pH 1.8; 7 M guanidine-HCl in 10 mM Tris-HCl; high pH DW, 13.5; and low pH DW, 0.5; and immunoreactivity was assessed by dot blots.

These experiments lacked specificity, so the procedure was refined. Lyophilized test extract was resuspended in PBS to a final protein concentration of four mg/ml. Trypsin, protease XIV, and pronase in PBS (final concentration 200 µg/ml) were added to 150 µl aliquots and allowed to digest (37°C, 3 hr, pH 7.2). Samples were vacuum centrifuged to dryness (2 hr), resuspended in 50 µl sample buffer, boiled, and run on PAGE as above. Gels were blotted as above, but before blocking were subjected to periodate oxidation (Woodward *et al.*, 1985). Blots were incubated in 20 mM sodium metaperiodate in 50 mM sodium acetate buffer (pH 4.5), or acetate buffer alone (negative control), in the dark (21°C, 1 hr). Blots were reduced in 50 mM sodium borohydride in PBS (21°C, 30 min), washed thrice in PBS, then probed.

Endoglycosidase F digestion was done on lyophilized test extract rehydrated to a final protein concentration of one mg/ml in: 50 mM potassium phosphate, 20

mM EDTA, 0.5% Triton X-100, 0.2% SDS, pH 6.5. This solution was then heated in a boiling water bath for 2.5 minutes. Three units of endoglycosidase F (Boehringer Mannheim) were added to the antigen solution to a final volume of 150 μ l, and incubated (37°C, 16 hr). The control was incubated with PBS. Samples were concentrated to dryness in a vacuum centrifuge, resuspended in 50 μ l sample buffer, run on PAGE and western blotted as described above.

To determine the epitope's sensitivity to heat denaturation, lyophilized test extract (rehydrated at four mg/ml in PBS) was held at 100°C (in a boiling water bath). Ten μ l samples were taken at 0, 2, 4, 8, 16, 32 and 64 minutes of incubation and were dotted onto nitrocellulose and probed as above.

Effect of developmental inhibitors on epitope expression

In all inhibitor experiments 200 ml cultures were grown at a density of 1000 embryos/ml. Except for the tunicamycin culture, embryos were incubated in the inhibitors until they were harvested for fixation. Specimens were fixed at 48 hours and 72 hours, and probed with Sp12 and NRH as outlined in Chapter 3.

Beta-aminopropionitrile (β APN) was added to cultures during cleavage to a final concentration of 0.5 mM. Cis-OH-proline (final concentration 100 μ M) incubated embryos were also probed. The inhibitor p-nitrophenyl- β -D-xyloside was added to cultures following fertilization to a final concentration of two mM.

For sulphate-free culture eggs were fertilized in FSW, then washed with (five times) and grown in sulphate-free artificial seawater (SFSW, MBL formula with magnesium chloride replacing the magnesium sulphate). Tunicamycin (1000X in methanol) was added to cultures after twelve hours to a final concentration of one μ g/ml. At 24 hours the embryos were washed three times in FSW to remove excess tunicamycin.

Controls were grown at the same density in FSW or in FSW with an equivalent amount of methanol as the experimental cultures.

Membrane and ECM isolation

Live cell assays were performed on dissociated blastulae, prisms, and plutei. Embryos and larvae were centrifuged (200g, 5 min), suspended in 25 volumes of calcium-magnesium-free-seawater with 100 μ M EDTA (CMFSW) (0°C, 10 min), then centrifuged (200g, 5 min) and resuspended in CMFSW again. This was repeated a third time, and the dissociating larvae were pipetted in and out of a pasteur pipette to resuspend them, then poured through 50 μ m Nitex to mechanically disrupt basal laminae and cell aggregates. The final cell suspension was centrifuged (800g, 5 min), resuspended in FSW, and incubated with primary antibody ascites diluted 1:300 (12°C, 1 hr). Cells were washed three times in FSW, then incubated in RAMIG-FITC diluted 1:50 (12°C, 30 min). After washing they were viewed under epifluorescence. Trypan blue was added to the final FSW suspension to determine the viability of cells.

Extracellular matrix was dissolved off embryos and larvae by glycine/EDTA treatment (McClay *et al*, 1977, Kane, 1973), or by CMFSW/EDTA treatment. Both solutions gave qualitatively the same result. About one ml of packed embryos or larvae were resuspended in 10 volumes of either 1.1 M glycine with 2 mM EDTA, or CMFSW with 2 mM EDTA (both containing the protease inhibitor cocktail) and briefly incubated (0°C, 15 min). They were then centrifuged (400g, 3 min), resuspended in another 5 volumes of extraction solution (0°C, 15 min), and centrifuged (400g, 5 min). Supernatants were combined and ultracentrifuged (150,000g, 60 min), dialyzed against DW (12 hrs, 4°C), lyophilized, and stored at -20°C. Cells from the above procedure were washed twice in CMFSW (0°C), and once in MFSW. Cells were incubated (0°C, 10 min) in 3 volumes of concanavalin A buffer: 1 μ m concanavalin A, 0.25 M sucrose, 5 mM Tris-HCl, 25 mM KCl, 2 mM magnesium sulphate, pH 8.0; to stabilize the plasma membrane. Cells were washed twice in the sucrose-Tris buffer, Dounce homogenized, and centrifuged (1250g, 10

min). The pellet was washed in the sucrose-Tris buffer, protease inhibitors were added and it was stored at -20°C . CMFSW extracts and membrane pellets were suspended in 1 ml IPAB and immunoprecipitated and western blotted as described above.

Extraction of membrane bound antigen

Plutei were dissociated by rinsing in CMFSW, incubating in 1.1 M glycine, and washing thrice in CMFSW. Supernatants were discarded and the cells lysed in LW (0°C , 30 min), then centrifuged (20,000g, 1 hr) and the pellet used for extractions. Extraction media included: DW, 1.1 M glycine, 100 mM sodium carbonate (pH 9.5), 2.5% butanol in DW, 50% butanol in DW, 95% ethanol with 10% acetic acid, 1% Triton X-100 in DW, 1% Triton X-114 in DW, and 1% sodium deoxycholate in DW. Three ml of solvent were added to about 100 μl of membrane preparation and extractions carried out at 4°C for one hour. The Triton X-114 extraction followed Bordier (1981); detergent was condensed (60°C , 25 min) and separated from the aqueous phase by centrifugation (900g, 10 min).

Samples were centrifuged (20,000g, 4°C , 2 hr) and supernatants frozen and lyophilized to about 20% of their original volume. Positive supernatants were ultracentrifuged (150,000g, 2 hr) to remove membrane micelles. Ten μl of each supernatant (aqueous and detergent phases from Triton X-114 extraction) and each pellet (suspended in an equal volume of sample buffer) were dotted onto nitrocellulose, dried and probed as in the western blotting procedure. In some cases extracted cells were washed in FSW and examined with indirect immunofluorescence to assess retention of the antigen.

Cellular affinity of the antigens

Cell populations from dissociated early plutei (96 hr PF) were separated using Nitex mesh. Two ml of packed larvae were dissociated in CMFSW as outlined above and the cell/basal lamina bag (BL bag) suspension was poured through 20

μm Nitex mesh until it became clogged. The BL bags were then gently washed from the Nitex, and the procedure repeated. After the whole suspension was separated the cells were centrifuged and resuspended in FSW, and BL bags were washed in FSW twice, settling at 1g between washes. The pour-through contained >99% single cells, and the BL bags were also >99% pure (free of single cells). The BL bags were very tough and no grossly broken bags were seen in the purity screens.

Aliquots of the separated fractions and whole embryos were fixed in 4% paraformaldehyde for immunofluorescence. The remainder was centrifuged (1000g, 10 min) and the pellets suspended in six volumes of sample buffer, then run on PAGE and western blotted as above.

Spicule growing primary mesenchyme cultures were produced from dissociated hatched blastulae cells following the method of Mintz, *et al.*, (1981). Briefly, embryos were dissociated in three washes of CMFSW. After resuspension at 10^7 cells/ml in FSW (with 0.3 U/ml penicillin and 0.5 $\mu\text{g}/\text{ml}$ streptomycin) containing 2% horse serum, cells were allowed to settle in 10 cm plastic petri dishes (14°C, 12 hr). Dishes were then washed by aspirating off the supernatant and gently applying a stream of FSW to the bottom of the petri dish. This was repeated twice and adherent cells were cultured for another three to five days (14°C) in FSW with pen/strep and 2% horse serum.

Cells and spicules were harvested by vigorous washing of the petri dishes, and centrifugation (1,000g, 10 min) of the resultant suspension. The pellet was dissolved in four volumes of 1.25X sample buffer, then run on PAGE and western blotted as above.

RESULTS

Ontogeny of the antigens by western blot analysis

Crude extracts

Coomasie blue stained polyacrylamide gels of samples from a series of developmental stages show qualitative and quantitative changes in protein composition through time (Fig. 10). When similar samples are western blotted and probed with Sp12 a number of immunoreactive bands are apparent (Fig. 11). Comparing the pattern of Sp12 antigens to the pattern of all the proteins in the samples shows that some of the prominent immunoreactive bands do not have Coomassie stained analogs, especially the bands with an M_r of greater than 97K. In general, each sample has multiple immunoreactive bands, and the pattern of both immunolabelled and Coomassie stained bands change, exhibiting a general decrease in M_r through development. The resolution of bands is poor, so a precise description of band patterns is difficult, however, broad differences are easily recognized.

A prominent band of immunoreactivity extends from the stacking gel - resolving gel interface down to about 200K in the unfertilized egg. This band disappears after fertilization, although discreet heavy bands are present in the embryonic samples. While the egg has a number of faint lighter bands, two or three bands at 100K to 180K are prominent, and remain prominent in embryos up to the prism stage. At early pluteus (96 hr PF) another broad change in pattern occurs. In feeding larvae bands at 90K and 110K replace the three heavier bands, although a faint band at 185K is still visible. In all samples a diffuse band at 70K is present, as is a complex of 35K to 45K.

Identical blots probed with NRH show no immunoreactive bands.

Immunoprecipitates

In order to increase the signal and resolution of immunoreactive bands, samples were immunoprecipitated before western blotting. Seven developmental stages, including egg, embryo, larva, and adult were immunoprecipitated, and then western blotted (Fig. 12a). Unfertilized eggs contain immunoreactive material of molecular weight greater than 170K which is unresolved at the top of the gel. Eggs also have faint bands at 120K and 70K.

At 24 hours (hatched blastula), the high molecular weight material is resolved into discrete bands. Two distinct bands of greater than 200K are present in all embryonic, larval, and adult samples. It is not possible to assign an M_r to these bands because they do not penetrate the gel far enough. Two bands, at 185K and 170K are present in all subsequent larval stages and the 185K band is prominent in the adult test. A band at 160K is present only in the 24 hour embryo and adult test. The 120K band seen faintly in eggs is much more pronounced at 24 hours. A faint new band at 35K is apparent in the 24 hour sample and is present through to the pluteus.

By 48 hours (full gastrula) the 160K band has disappeared and two new bands at 200K and 140K appear which persist until late pluteus. The 140K band is prominent, but its intensity decreases in 120 hr and 20 day plutei.

Early plutei (84 hr PF) exhibit a pattern similar to gastrulae except the 120K band is broader and a band at 90K is present. This faint 90K band continues to be expressed in plutei.

At 120 hours (four arm pluteus) there are discrete bands at 105K and 120K, and the 200K and 140K bands have lost intensity. The pattern in older plutei is the same as in 120 hour larvae, although it is fainter.

The adult test sample has six bands in common with larval samples: two greater than 200K, and one each at 185K, 160K, 140K, and 105K. Unique bands at 110K, and 75K are not shared with any larval samples.

Samples immunoprecipitated with NRH and probed with Sp12 reveal no Sp12 immunoreactive bands. Similarly, Sp12 immunoprecipitated samples probed with NRH are negative for antigen bands (Fig. 12b). This blot illustrates the secondary antibody recognition of immunoprecipitating ascites antibodies - heavy chains and light chains are labelled at about 50K and 25K in both the NRH and Sp12 probed blots.

The same samples were blotted onto nitrocellulose again and probed with concanavalin A-HRP. This blot (Fig. 12a) reveals concanavalin A reactive sugars in the unfertilized egg sample. Distinct bands at about 215K, 170K, 120K, and 70K are labelled, as well as three faint bands between 70K and 120K. There is a faint band at 215K in all seven samples in addition to the clearly concanavalin A reactive IgG heavy chains from the immunoprecipitating antibodies. The NRH blot was stained with periodic acid/Schiff's reagent (PAS) and reveals exactly the same pattern as the concanavalin A labelling (Fig. 12b).

Reduced versus unreduced samples

Reduced and unreduced samples from 6 developmental stages were run on PAGE and western blotted (Fig. 13). As these are crude samples, the bands are not as clearly defined as in the immunoprecipitates of Fig. 12. Reduced-sample bands at 140K and 130K appear to be compressed into a broad band at 160K in unreduced samples. Antigens with lower M_r 's are present in both treatments, however, the unreduced antigens are not as prominent as the reduced ones. Unreduced samples have an additional band at about 235K, which is not apparent in the reduced samples. These results are consistent with the presence of disulphide bonds in these antigens which can reduce their mobility in native gels.

Proteolysis

The recognition of multiple antigens by Sp12 could be due to a redundant epitope or to proteolysis of a single high molecular weight precursor. To determine if artifactual proteolysis causes the multiple bands, solubilized 96 hour samples were held under various conditions before immunoprecipitation and western blotting (Fig. 14). With all treatments, the pattern and intensity of the multiple antigens is identical. In addition, this pattern is very similar to that of the 84 hour sample of Fig. 12a indicating that the number and M_r of labelled bands is reproducible.

Epitope determination

As Sp12 labels multiple antigens, it is important to establish the chemical nature of the redundant epitope. The dot-blot after enzyme and chemical treatments provide an initial analysis. Ribonuclease A, hyaluronidase, chitinase, and 7M guanidine-HCl have no effect on Sp12 immunoreactivity, while proteases and extremes of pH markedly reduce it. Periodate treatment at pH 1.8 reduces immunoreactivity while the same reagent at pH 7.2 has no effect on the epitope.

The dot-blot analysis indicates that the epitope could be a peptide, however, it is possible that instead of destruction of a protein epitope, proteolysis causes loss of a carbohydrate epitope as a peptide-carbohydrate fragment. This possibility was addressed with a more rigorous enzymatic and periodate treatment of the test extract. Western blots of PAGE separated antigens provide more details of enzyme effects in these experiments (Fig. 15; Fig. 16). Consistent with the dot-blot experiments, trypsin, protease XIV, and pronase all eliminate immunoreactivity. However, as shown by amido black staining of these blots, protease treatments also eliminate almost all proteins (and any post-translational modifications to them). In these preparations 20 mM and 40 mM periodate reduce Sp12 immunoreactivity, but neither concentration eliminates it (Fig. 15).

The partial reduction of immunoreactivity by periodate indicated that enzymatic cleavage of carbohydrates from the antigens could help resolve the nature of the epitope. Small aliquots of test extract were digested with endoglycosidase F which cleaves N-linked carbohydrate chains from their protein cores. Endoglycosidase F eliminates immunoreactivity of all five bands present in the control (Fig. 16). Only a minor change in the pattern and content of proteins in the sample is evident following this treatment. As a positive control, concanavalin A reactivity is also eliminated in treated samples, indicating the endoglycosidase F does cleave carbohydrate moieties from glycoproteins in this sample.

Incubation of extracts at 100°C for up to 64 min does not affect immunoreactivity, although the pattern of antigen dots appears more condensed in boiled samples (Fig. 17). The pH of this solution remains constant at 6.5 - 7.0 over the course of the experiment. Controls taken before incubation and following 64 min at 37°C show the same level of immunoreactivity.

Effect of gastrulation inhibitors on epitope expression

β APN inhibits collagen formation and prevents gastrulation in the sea urchin embryo (Butler *et al.*, 1987; Wessel and McClay, 1987). In 48 hour embryos incubated in β APN mesenchyme do migrate, and cells disperse around the blastocoel, although no organization of them is apparent. This inhibitor reduces the number of mesenchyme cells and Sp12 immunoreactive cells, but does not eliminate immunoreactivity (Fig. 18c,d). Cis-OH-proline also inhibits collagen formation and gastrulation. This reagent is a stronger inhibitor of mesenchyme ingression and migration and causes a greater reduction in the number and intensity of Sp12 immunoreactive cells.

The reagent p-nitrophenyl- β -D-xyloside inhibits the linking of carbohydrate moieties to protein cores in proteoglycan synthesis. While this inhibitor clearly disrupts gastrulation, in 72 hour embryos mesenchyme have migrated within the

blastocoel, and rudimentary archenterons are occasionally observed. These mesenchyme cells are fully immunoreactive when probed with Sp12 (Fig. 18e,f).

Embryos grown in seawater devoid of sulphate ions develop normally up to the blastula stage, but fail to gastrulate, or have delayed gastrulation with abnormal archenterons. Embryos cultured in SFSW for 48 hours (controls are full gastrulae) show two patterns of Sp12 immunoreactivity. The usual result is similar to that seen with tunicamycin - hyaline layer labelling, but negative cellular fluorescence. In one experiment, however, an interesting pattern was observed. The embryos did not gastrulate, but primary mesenchyme formed a ring in the vegetal pole, and one to five cells migrated into the animal hemisphere. These cells in the animal hemisphere expressed Sp12 antigens, while the primary mesenchyme in the vegetal hemisphere did not (Fig. 18g,h). This result has not been repeated, presumably because it requires an exact amount of sulphate - enough to allow some mesenchyme migration, but not gastrulation. Without repeated results no conclusion can be drawn, but this appears to be a case where migration into the animal hemisphere (and receipt of a differentiation signal there) is required for epitope expression.

Tunicamycin inhibits the first step in N-linked carbohydrate side-chain synthesis, thus blocking N-linked glycoprotein production. Tunicamycin treated 48 hour embryos are severely affected: none of them gastrulate and mesenchyme remains in an unorganized clump at the vegetal pole. None of these cells are Sp12 immunoreactive. Hyaline layer immunoreactivity, however, is present above control levels in these embryos (Fig. 18i,j). When allowed to develop longer the mesenchyme start to fragment and vesiculate, but no change in immunoreactivity is observed.

Membrane-associated and extracellular antigen separation

A live cell assay was performed to confirm that the Sp12 mesenchymal antigens are cell surface molecules. Hatched blastulae (24 hr), prisms (72 hr), and plutei (120 hr) were dissociated with CMFSW and probed with Sp12. Dissociated live cells from all three stages are immunoreactive, but two patterns of cellular labelling are evident: asymmetric and uniform (Fig. 19). This immunoreactivity is still apparent in specimens that have been washed repeatedly in CMFSW or 1.1 M glycine. The pattern of immunoreactivity does not change if the cells are incubated, with bound antibody, for a further three hours. The antigens do not "cap".

Presumptive mesenchyme cells in the prism and pluteus dissociates are intensely and uniformly labelled (Fig. 19c-f). This includes cells within intact basal lamina bags as well as unenclosed cells (pigment cells or mesenchyme from broken basal lamina bags). An unexpected finding is the asymmetric cell surface immunoreactivity of the 24 hour cells. The dissociated cells clearly show a discrete patch of plasma membrane that retains antigens after removal of the hyaline layer. Again, this pattern of immunoreactivity does not change with extended incubations. This patch of immunoreactivity is also present on presumptive ectodermal cells at 72 hours and 120 hours, but the intensity of immunofluorescence fades considerably in older larvae (Fig. 19).

The *in situ* ontogeny, tissue specificity, and live cell labelling results indicate that Sp12 labels antigens both in the ECM and on the cell surface. To further test this hypothesis, extracellular and membrane associated antigens from blastulae (24 hr), prisms (72 hr), and plutei (120 hr) were separated, immunoprecipitated, and western blotted (Fig. 20). The 24 hour CMFSW extract has a band above 200K with a faint band just below it. Very faint bands at 180K and 85K are detectable in the original blot. The 24 hour membrane fraction has a faint doublet above 200K corresponding to the CMFSW bands, a more prominent 180K band and a diffuse

85K band. At 72 hours the CMFSW extract has one prominent and two minor bands above 200K, and bands at 200K, 180K, and 85K. The membrane fraction has a prominent band at 180K, with fainter bands above 200K, at 145K, 120K, and 85K. In the 120 hour CMFSW extract, the pattern of antigens is almost identical to the 72 hour sample, although fainter. The 120 hour membrane fraction exhibits a prominent 180K band as well as bands at 145K and 85K. All samples have immunoprecipitating mouse ascites antibodies, hence bands at 45/50K and 25K are present in both Sp12 and NRH blots and represent heavy chains and light chains respectively.

Membrane bound antigen extraction

Lysed cells from dissociated early plutei were used in extraction experiments designed to assess whether the membrane bound antigens are integral or peripheral components. Following incubation of crude membrane preparations in various solvents, supernatants and pellets were dotted onto nitrocellulose and probed for immunoreactivity (Fig. 21).

Distilled water and 1.1 M glycine release a small amount of antigen (immunoreactivity) from the crude membranes into the supernatant. Sodium carbonate (100 mM, pH 9.5) solubilizes an intermediate amount of antigen. The 2.5% butanol solution, two phase butanol/DW extraction, and 95% ethanol do not solubilize any detectable antigen. Little antigen is present in the supernatant of the 1% Triton X-100 extraction, and antigen is not present in the detergent phase of 1% Triton X-114, however, a large amount is present in this solution's aqueous phase. Sodium deoxycholate (1%) solubilizes the maximum amount of antigen (equivalent to the levels attained using heated sample buffer).

Dissociated larvae treated with 1.1 M glycine and 2.5% butanol were washed in FSW and probed with Sp12 as in the live cell assay. Treated cells within the basal lamina and free in solution retained the cellular staining characteristic of Sp12.

The pellets of all the extractions retain some immunoreactivity, which is solubilized by sample buffer. The pellets vary in texture and compactness, the detergent extracted pellets being more gelatinous and voluminous. Each pellet was suspended in an equal volume of sample buffer resulting in varied antigen concentrations.

Cellular affinity of the antigens

Sp12 antigens can be further classified by their expression on specific cell types. There are considerable differences in the pattern of antigens present on cells of the epidermal fraction and on the mesenchyme within the basal lamina. The two patterns of antigen appear to complement each other, and taken together give the same pattern and number of bands as is present in the whole embryo fraction (less the CMFSW extractable bands) (Fig. 22). The endoderm/mesoderm fraction (basal lamina bags) has only three bands recognized by Sp12. These are at 185K, 120K, and faintly, at 80K. The ectoderm fraction by contrast, contains six or seven difficult to resolve bands, at 200K, 170K, 140K, 100K, 90K, 80K, and 45/35K. The whole embryo sample has bands at over 200K, 200K, 180K, 170K, a smear from 140K to 120K, 100K, 90K, 80K, and 45/35K.

Skeletogenic mesenchyme cultures were also probed with Sp12. Five or six bands are apparent at 200K, 185/170K, 140K, 120/105K, and 90K (Fig. 22).

NRH probed blots of these samples are devoid of any labelled bands (Fig. 22).

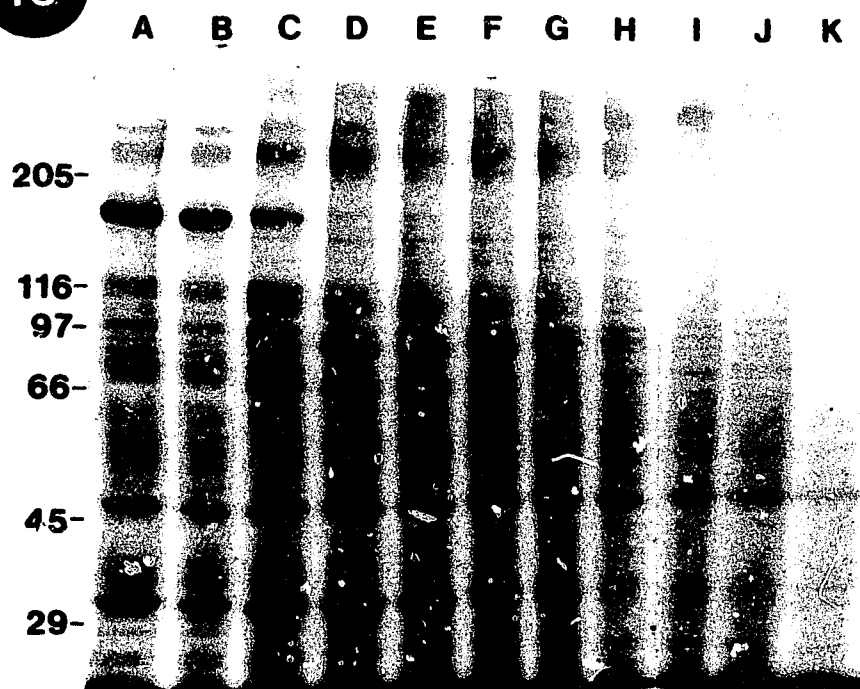
Confirmation of the reproducibility of the banding patterns observed in these experiments is provided by comparisons of several 72 hr - 96 hr samples. A composite of lanes, each from a different experiment, shows that the pattern of bands in early plutei is reproducible (Fig. 23). There are slight differences in the intensity and number of some bands between samples, but there is a wide range of

sample treatments represented. Reproducible, analagous bands are indicated with numbered arrowheads.

Figure 10. PAGE separated proteins from 11 developmental stages of *S. purpuratus*. Coomassie blue stained 7.5% gel. A) 0 hr, unfertilized egg. B) 1.5 hr, single cell zygote. C) 18 hr, blastula. D) 32 hr, mesenchyme blastula. E) 43 hr, half gastrula. F) 50 hr, full gastrula. G) 60 hr, early prism. H) 72 hr, late prism. I) 96 hr, early pluteus. J) 5 d, pluteus. K) 8 d, four arm pluteus. This gel is not quantitative, but shows qualitative changes in the proteins present through the first week of sea urchin development. In general, it appears that the number of proteins decreases with development, so that larvae are simpler (in protein makeup) than eggs. The M_r 's of molecular weight standards are indicated.

Figure 11. Western blot of samples from a developmental series, probed with Sp12. A) 0 hr, unfertilized egg. B) 12 hr, morula. C) 24 hr, hatched blastula. D) 32 hr, mesenchyme blastula. E) 42 hr, quarter gastrula. F) 50 hr, full gastrula. G) 60 hr, early prism. H) 76 hr, late prism. I) 96 hr, early pluteus. J) 5 d, pluteus. K) 7.5 d, four arm pluteus. A mass of very heavy antigen present in the unfertilized egg is resolved into discreet, generally lighter, bands after fertilization. Groups of antigens at about 40K and 70K are shared by all samples. From 12 hours through to 76 hours two or three bands at 100K to 180K are labelled. The 96 hr - 7.5 d samples have bands at 90K and 110K, replacing the heavier triplet of non-feeding embryos. The overall trend is toward fewer bands of lighter M_r , a pattern similar to that of proteins in general through development. The M_r 's of molecular weight standards are indicated. An identical blot probed with NRII is absolutely clean, no nonspecific bands are apparent.

10



11

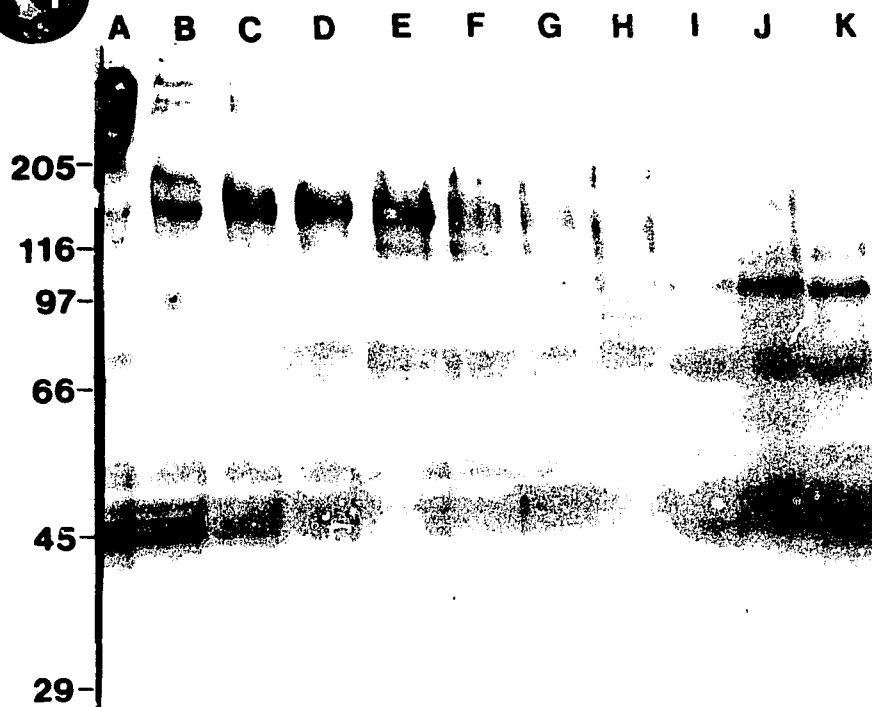


Figure 12. Western blot of immunoprecipitated samples from a developmental series. All samples are from the same batch of embryos except the 20 d sample - the adult test is from the spawned female. Samples were immunoprecipitated with Sp12 then run on PAGE and western blotted. **a)** A-G is the blot probed with Sp12. A) 0 hr, unfertilized egg. B) 24 hr, hatched blastula. C) 48 hr, full gastrula. D) 84 hr, early pluteus. E) 5 d, four arm pluteus. F) 20 d, six arm pluteus. G) Adult test scraped clean of all soft tissue. The pattern of antigen banding is outlined in the text, but it is clear that there are multiple antigens present, and the M_r of antigens changes through development. The M_r 's of heavy molecular weight standards are indicated. H,I) An identical blot probed with concanavalin A. H) 0 hr, unfertilized egg. I) Adult test sample. All the other samples are identical to the adult test sample and are not included. **b)** A-G This blot is identical to a), but was probed with NRH. All the lanes correspond to those in a). Mouse immunoglobulins from the immunoprecipitating ascites are detected in both blots. Heavy chains comprise three bands at 50K, and light chains form a doublet at 25K. The M_r 's of heavy molecular weight standards are indicated. H,I) The NRH blot stained with periodic acid Schiff's reagent to detect reducing sugars. H) 0 hr, unfertilized egg. I) Adult test sample. The pattern of sugars detected by PAS is identical to that detected by concanavalin A. The antibody bands are immunolabelled from the NRH labelling.

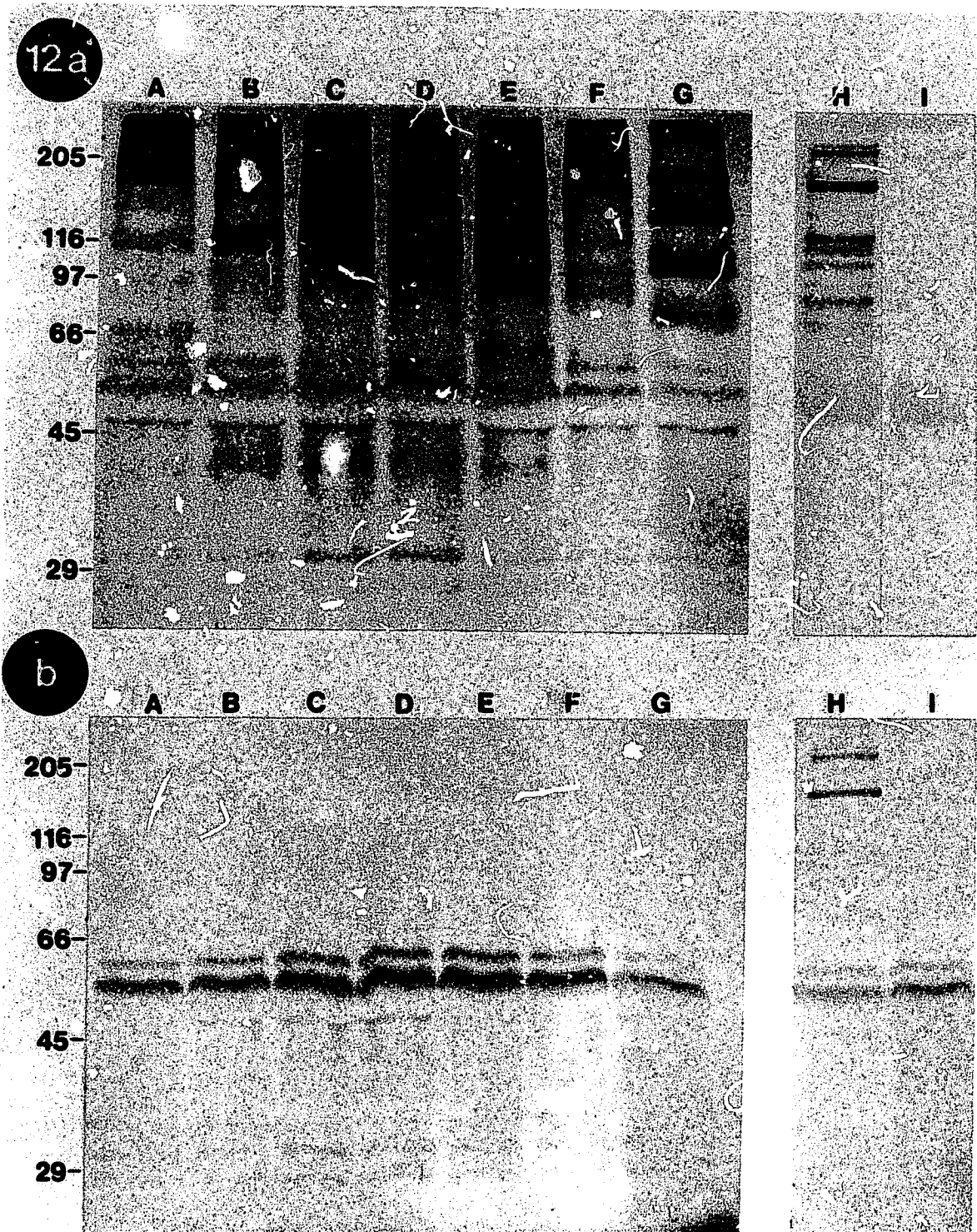
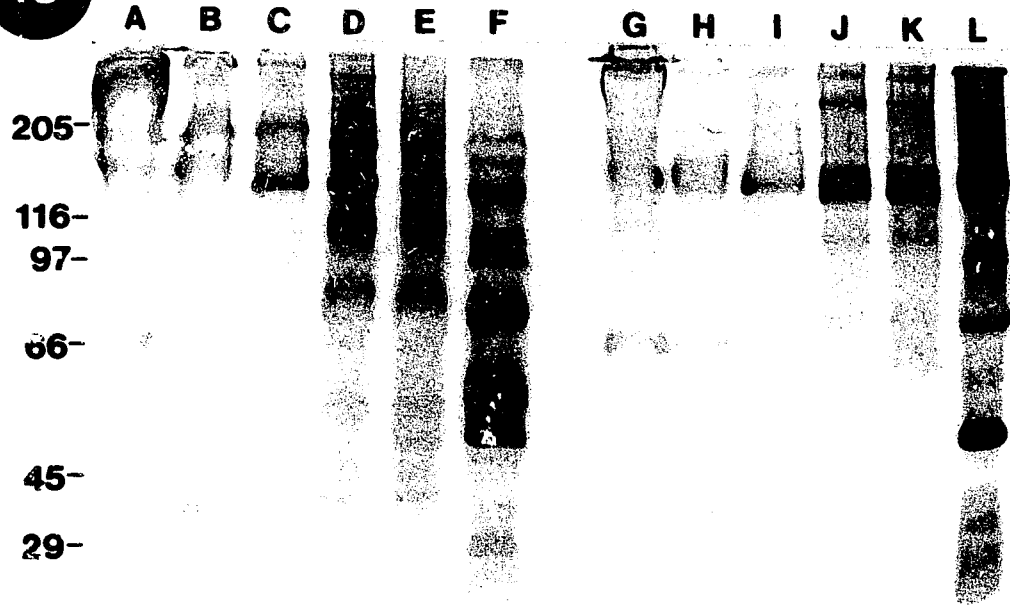


Figure 13. Patterns of antigens under reducing and nonreducing conditions. A-F) Reduced samples. A) 0 hr, unfertilized egg. B) 24 hr, hatched blastula. C) 48 hr, full gastrula. D) 80 hr, early pluteus. E) 5 d, pluteus. F) Adult test, scraped clean of all soft tissue. These are not immunoprecipitated samples, so the bands are not as tight as those in the Fig. 14, however, an analogous pattern of bands is apparent. G-L) Nonreduced samples, mercaptoethanol was omitted from the sample buffer. Lanes are identical to those in A-F). There are shifts in M_r (heavier) of some bands, and fewer bands overall. The band at about 160K is the major component in all unreduced samples, while plutei have an additional band at about 235K. The M_r 's of prestained heavy molecular weight standards are indicated.

Figure 14. Artifactual proteolysis does not produce the multiple bands. Aliquots of 96 hr embryos were suspended in four volumes of immunoprecipitation assay buffer and held under various conditions before immunoprecipitating, running on PAGE, and western blotting. A-D) Blot probed with Sp12. A) 30 min at 21 °C, without protease inhibitors. B) 30 min at 21 °C, with protease inhibitor cocktail. C) 24 hr at 4 °C, without protease inhibitors. D) 24 hr at 4 °C, with protease inhibitor cocktail. There are no differences in the intensity or pattern of antigens, despite the wide range of treatments. E-H) A blot identical to A-D, probed with NRH. Immunoprecipitating antibody bands are apparent. The M_r 's of prestained heavy molecular weight standards are indicated.

13



14

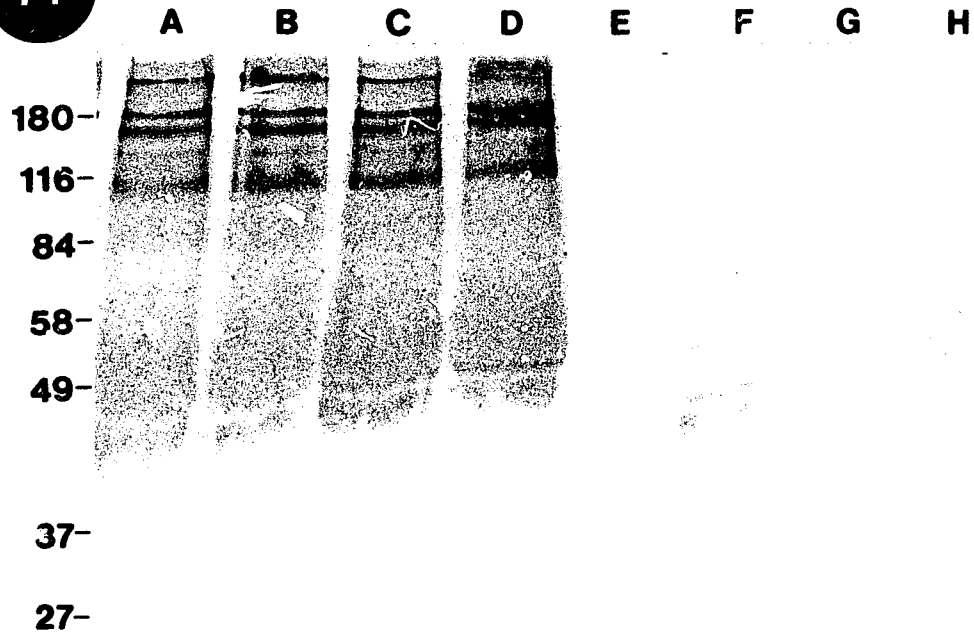


Figure 15. Proteolysis and periodate sensitivity of the Sp12 epitope. Crude adult test antigen (at 4 mg/ml protein) was utilized for all epitope determination experiments. Lanes A, C, and E were treated with PBS (negative control); lanes B, D, and F were treated with trypsin (200 μ g/ml, 3 hr, 37°C) before running on PAGE and western blotting. A, B) The blot was treated with sodium acetate and sodium borohydride, then washed in PBS before probing with Sp12. C, D) This blot was treated with 20 mM sodium metaperiodate in acetate buffer (1 hr, 21°C), then reduced in sodium borohydride and washed in PBS before being probed with Sp12. E, F) This blot was stained with amido black after being treated like blot A, B. NRH probed blots showed no bands. The M_r 's of prestained heavy molecular weight standards are indicated.

Figure 16. Endoglycosidase F treatment of the Sp12 antigens. Lanes A, C, and E were treated with PBS (negative control). Lanes B, D, and F were treated with endoglycosidase F (16 hr, 37°C) before running on PAGE and western blotting. A, B) This blot was probed with Sp12. Endoglycosidase F eliminated almost all immunoreactivity (lane B). C, D) Concanavalin A probing of the same samples reveals that the endoglycosidase F does cleave off carbohydrate sidechains (lane D). E, F) Amido black stained blots show almost identical patterns of protein bands in the two treatments. There are minor alterations in the intensity of some bands following treatment. Identical blots probed with NRH showed no bands. The M_r 's of prestained heavy molecular weight standards are indicated.

Figure 17. Boiling does not reduce Sp12 immunoreactivity. Antigen solubilized in PBS was held in a boiling water bath at pH 7.0. Ten μ l aliquots were dotted onto nitrocellulose at the times indicated and treated as western blots. The upper row was probed with Sp12, the lower row was probed with NRH and shows staining due to echinochrome pigment.

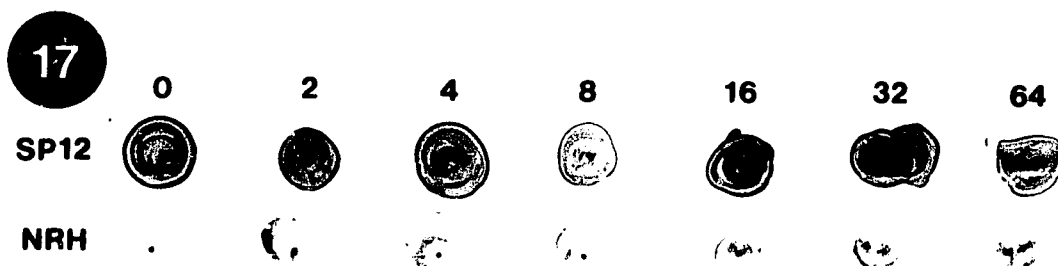
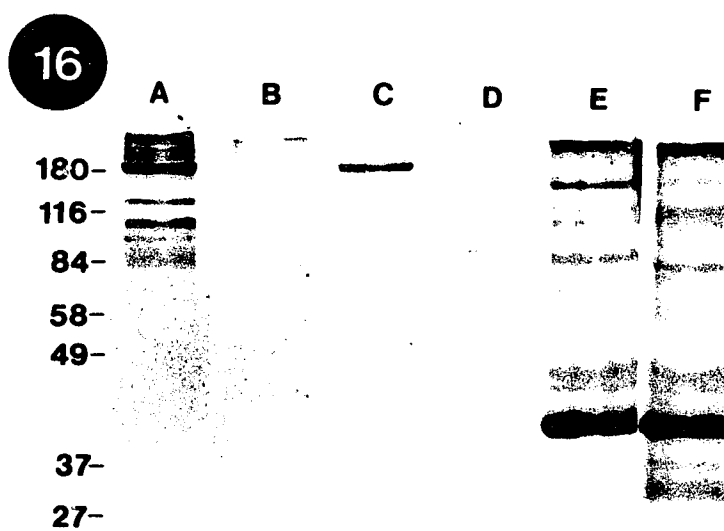
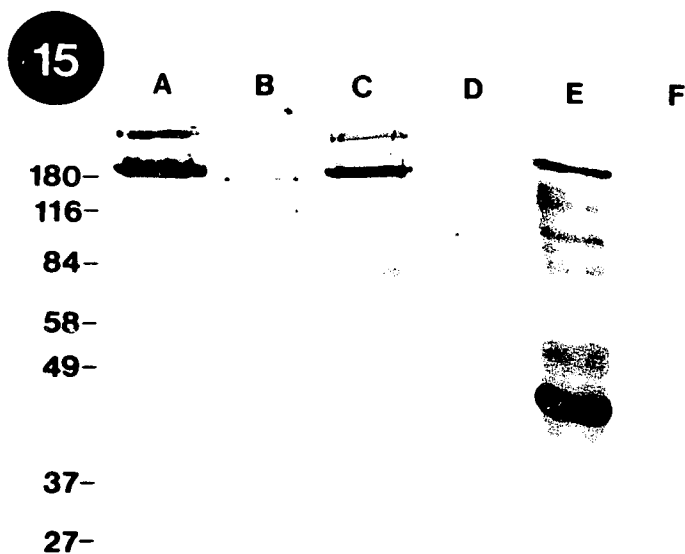


Figure 18. Effect of developmental inhibitors on Sp12 epitope expression. Indirect immunofluorescence micrographs are shown in a, c, e, g, i, and k with corresponding light micrographs in b, d, f, h, j, and l; a - i are Sp12 labelled while k is NRH labelled. **a, b)** Control specimen, grown in FSW for 48 hr. Hyaline fluorescence within the archenteron is not normally observed and may be a batch artifact. Otherwise the embryo is normally labelled with primary and secondary mesenchyme immunoreactive. **c, d)** β -amino proprionitrile (β APN) incubated, 48 hr embryo. The embryo is small and has not gastrulated, although mesenchyme within the blastocoel expresses the Sp12 epitope. **e, f)** p-nitrophenyl β -D-xyloside incubated, 72 hr embryo. This embryo is severely disrupted, likely due to prolonged incubation in the inhibitor. Despite this, it is clear that gastrulation is inhibited, and that the Sp12 epitope is expressed on mesenchyme in the blastocoel. **g, h)** Embryos raised in sulphate-free-seawater generally do not produce Sp12 positive mesenchyme, as in i). However, occasionally mesenchyme ingresses and migrates, which may trigger Sp12 expression on a subset of cells. In this 48 hr embryo, mesenchyme in the vegetal portion of the embryo are not Sp12 immunoreactive (arrow), but a few mesenchyme cells in the animal hemisphere are strongly immunoreactive (arrowhead). **i, j)** Tunicamycin incubated 48 hr embryo. This embryo has not gastrulated but mesenchyme has ingressed (arrow). None of these cells show any hint of Sp12 expression. The hyaline layer is immunoreactive, apparently because this Sp12 antigen is preformed in the unfertilized egg. **k, l)** NRH probed, 48 hr embryo grown in FSW (negative control). This is the NRH immunoreactivity seen with all inhibitor-incubated specimens as well; there is no nonspecific fluorescence associated with any of these specimens. The scale bar is 20 μ m.

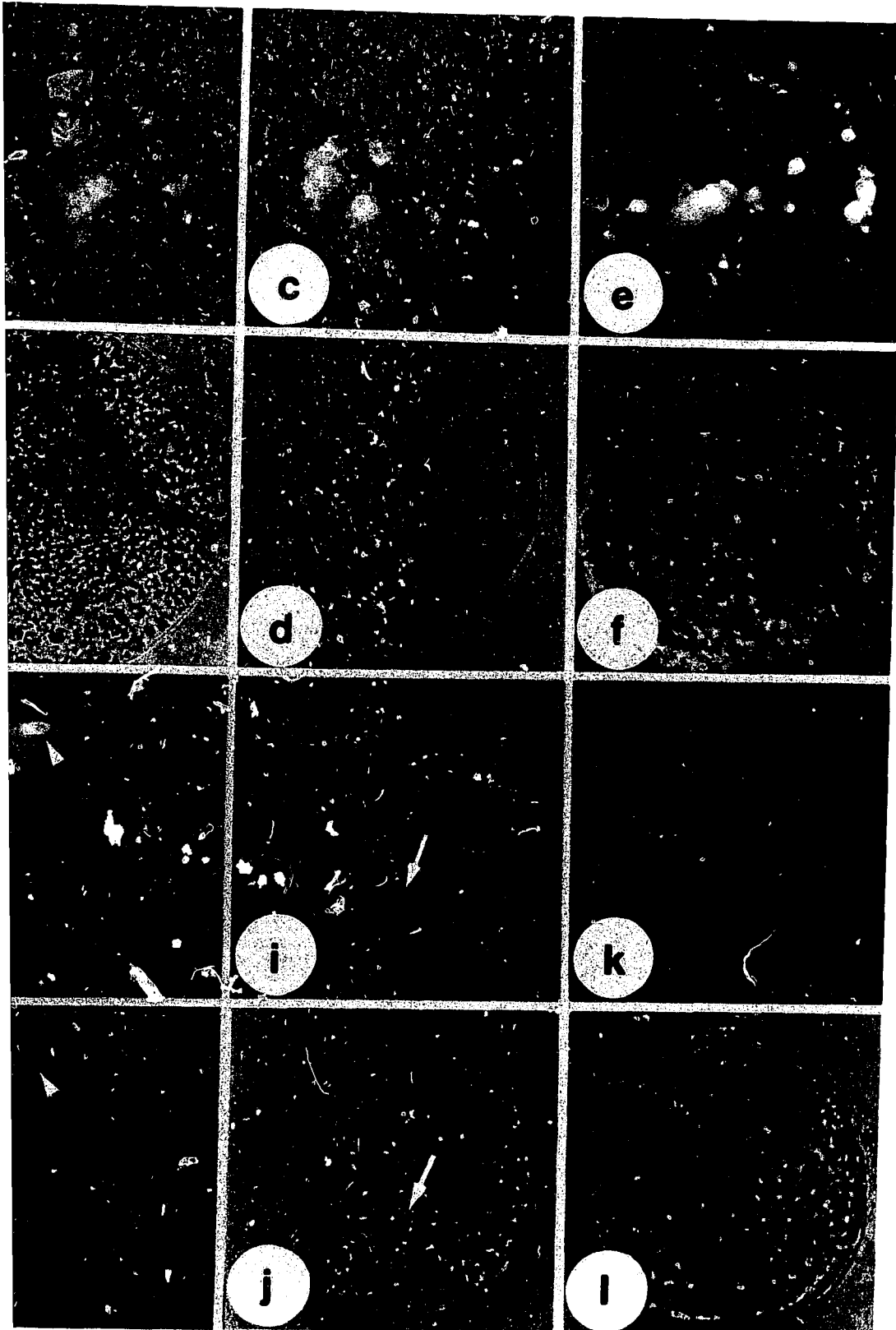


Figure 19. Sp12 labelling of live cells. Immunofluorescence micrographs are shown in a, c, and e with corresponding light micrographs in b, d, and f. **a, b)** Cells dissociated from hatched blastulae (24 hr embryos). Most cells have a distinct cap of antigen (arrowheads) which is present immediately after assay (1 hr after first addition of antibody). The pattern of labelling of cells from all stages does not change if cells are incubated a further 3 hr. **c, d)** Cells from dissociated prisms (72 hr embryos). A fraction of the cells are capped (arrowheads), but strongly fluorescent, uniformly labelled cells are more prominent (arrows). **e, f)** Cells from dissociated plutei (5 d larvae). Capped cells are rare and weakly reactive (arrowheads), while uniform, intense labelling is still apparent on some cells (arrows). Scale bar is 10 μ m.

Figure 20. Separation of ECM and membrane associated antigens. A, C, and E are ECM extracts, while B, D, and F are crude membrane pellets, G is a whole embryo extract. A - G are Sp12 labelled, while H - J are NRH labelled lanes corresponding to E - G. A, B) ECM and membrane fractions from 24 hr, hatched blastulae. C, D) ECM and membrane fractions from 72 hr embryos. E, F). ECM and membrane fractions from 120 hr larvae. G) The 120 hr whole embryo sample. Bands from lanes E and F appear to sum in this sample. H, I, J) NRH labelled blot, which shows immunoprecipitating antibody bands. M_r 's of prestained heavy molecular weight standards are indicated.

Figure 21. Dot blot analysis of antigen extraction from crude membrane preparations. Dots in the top row are supernatants, those in the bottom row are pellets (solubilized in sample buffer). A) Distilled water. B) 1.1 M glycine in DW. C) 100 mM sodium carbonate, pH 9.5. D) 2.5% butanol in DW. E) 50% butanol in DW, organic phase. F) 1% Triton X-100 in DW. G) 1% Triton X-114, aqueous phase. H) 1% sodium deoxycholate in DW. NRH probed dot blots are all negative.

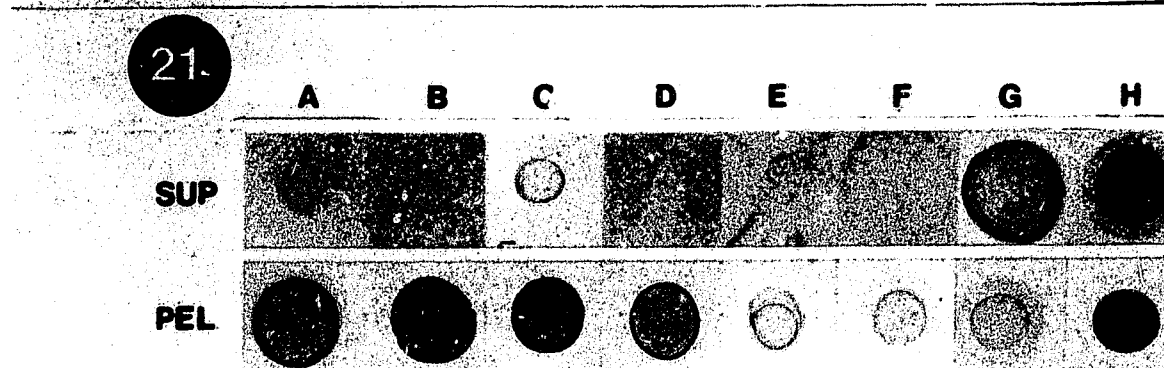
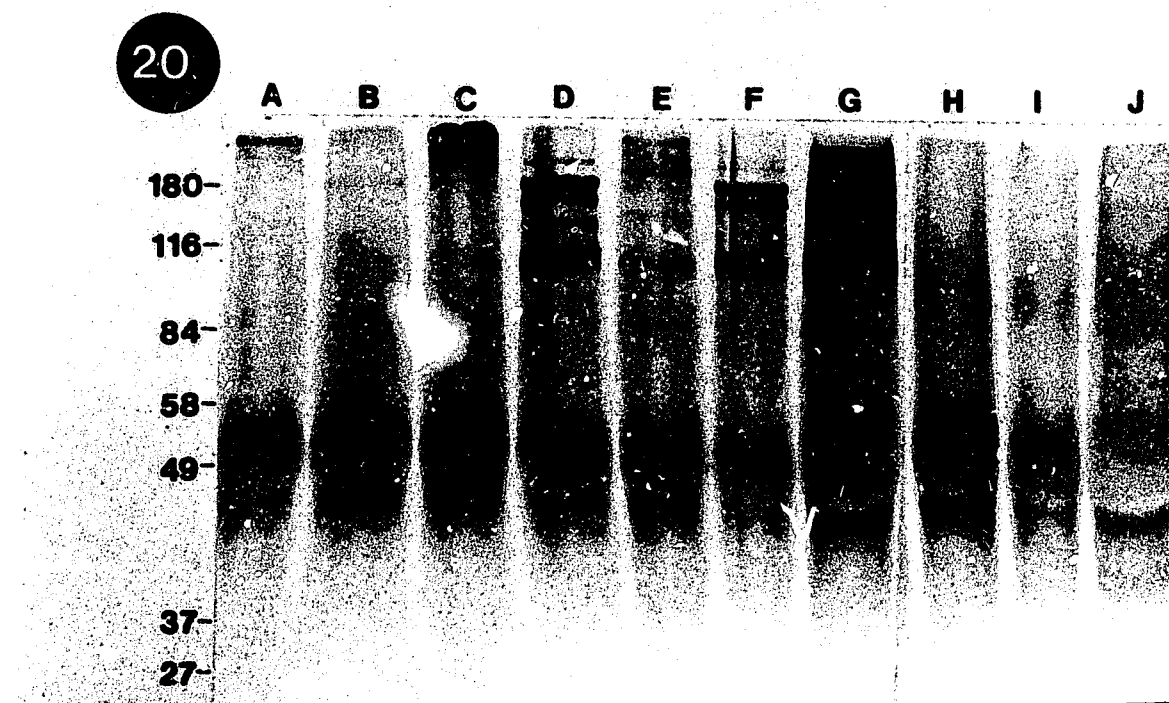
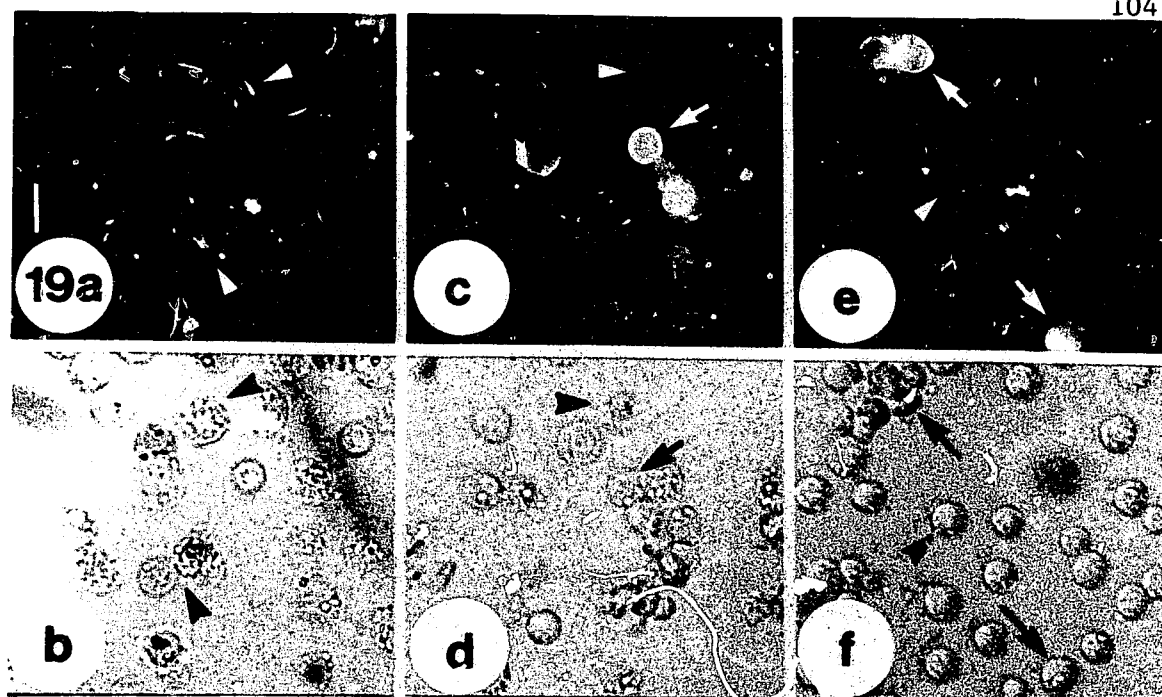
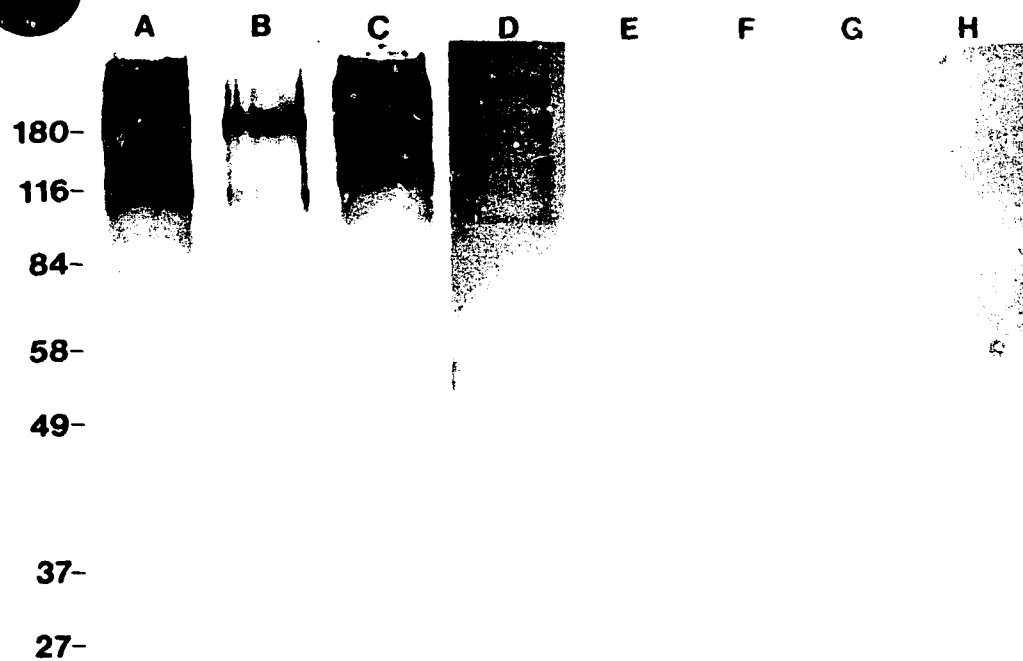


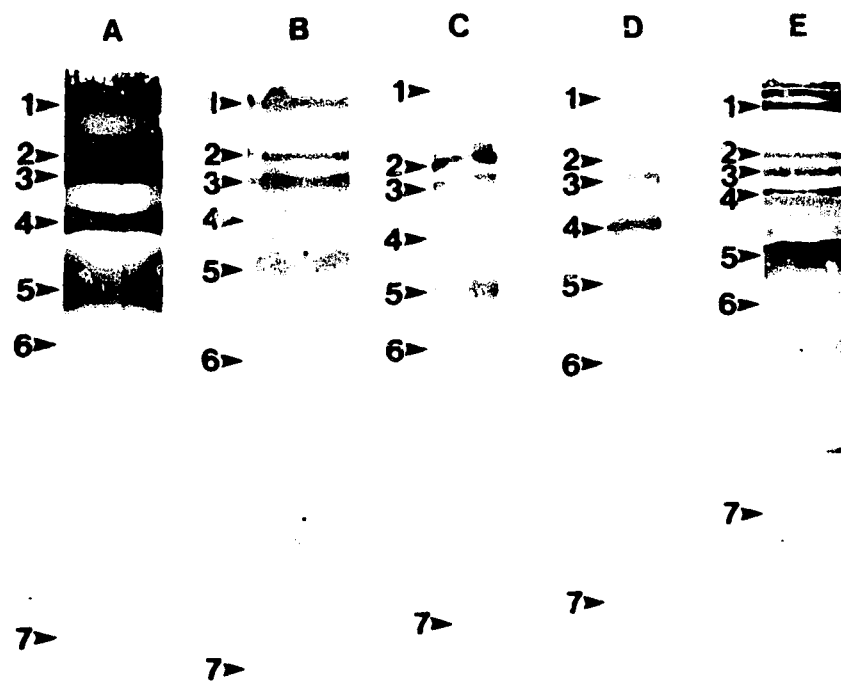
Figure 22. Antigens in cell fractions. Dissociated 96 hr embryos were poured through 20 μ m Nitex mesh separating ectodermal cells (including pigment cells) from basal lamina bags (endoderm and mesoderm). Primary mesenchyme cell cultures were harvested into sample buffer from petri dishes. Samples were not immunoprecipitated, but run directly on PAGE and western blotted. The bands are diffuse, but despite this, patterns can be discerned. Lanes A-D are Sp12 probed, while lanes E-H are corresponding NRH blots. A) Whole embryo extract. Though poorly resolved, the two cell fraction lanes appear to combine in the whole larva sample. B) Endodermal/mesodermal fraction - three bands are visible. C) Ectodermal fraction. Two dense bands and six faint bands are labelled. They appear to be complimentary to the bands in B. D) Cultured skeletogenic cells contain several antigens. Although the blot is underdeveloped, it is clear that at least six bands are Sp12 immunoreactive in these cultures. E - H) NRH probed blots show no evidence of immunoreactivity. M_r 's of prestained heavy molecular weight standards are indicated.

Figure 23. Composite of western blots from analogous developmental stages. This figure illustrates the degree of repeatability of this banding pattern. All of these samples received very different treatments, yet they all exhibit a similar banding pattern. Numbered arrowheads indicate seven analogous bands. A) 84 hr sample from Fig. 14. B) The 96 hr sample held at 21°C for 30 min with the protease inhibitor cocktail (Fig. 16, lane B). C) 96 hr sample from a previous immunoprecipitation. D) 80 hr sample from the reduced series (Fig. 15, lane D). E) 72 hr sample from another developmental series of immunoprecipitations. Molecular weight standards are not shown as they vary with each gel, samples are presented for qualitative comparison only.

22



23



DISCUSSION

The immediately striking result presented by Sp12 probed western blots is the multiplicity of bands labelled by this monoclonal antibody. On closer examination, the immunoreactive bands are not only numerous, they also vary in both intensity and apparent molecular weight through development. The complexities attendant on sorting out the Sp12 antigens have made interpretations difficult, but a number of different approaches were utilized to that end. The final result provides a working model for further investigations of this system.

Changing pattern of Sp12 antigen expression

The PAGE pattern of proteins through development appears to fade overall in older stages, with a greater proportion of lighter M_r antigens. This is likely due to a decreased amount of protein in these samples since lanes were equalized by solubilizing the same volume of embryos of each stage and older larvae are much less dense than early embryos. Comparing the crude protein result to that of the Sp12 probed western blot, Sp12 antigens do not appear to correspond to the prominent proteins, but they do show a generalized drop in apparent M_r through development. Immunoprecipitation prior to SDS-PAGE provides greater intensity and resolution of the Sp12 bands, and compared to the crude western blots results in much heavier labelling of the high M_r antigens. Perhaps these large antigens are more likely to be immunoprecipitated out of solution than small ones, or conversely, perhaps large antigens do not enter gels or are not detected on blots unless enriched by immunoprecipitation first.

There are four points of change in the western blot profiles through development: following fertilization, between blastula and gastrula, between prism and pluteus, and between pluteus and adult. Key features of these are noted below. Subtler aspects of the western blot patterns are difficult to interpret, as they are not consistently reproducible they are not considered (see Fig. 23).

Sp12 antigen is predominantly unresolved, with a high M_r , in the unfertilized egg. At 12 hr PF this mass of antigen is resolved into discrete bands. While direct evidence is not presented here, it is a reasonable hypothesis that this resolution of antigens is associated with fertilization and cortical granule exocytosis. In this model, the cortical granule antigens are chemically modified following their release into the external milieu, forming a part of the hyaline layer as a group of proteins of varied molecular weights. Concanavalin A labelling and PAS staining of developmental sequence western blots contributes further evidence of this chemical modification. Several bands are labelled in the unfertilized egg, while only one faint band at over 200K is evident in all subsequent samples. This must reflect either loss of carbohydrate groups during processing at fertilization, or loss of coprecipitated glycoproteins. Even if the glycoproteins so labelled are only coprecipitated, their absence in later stages indicates modification of the bound antigens.

There is growing evidence of enzymatic modification of cortical granule contents following fertilization. A specific proteolytic enzyme (Alliegro and Schuel, 1988) and β -1,3-glucanase (Truschel *et al.*, 1986) are localized to cortical granules, released to the perivitelline space at fertilization, and presumably degrade their respective substrates. Degradation of the cortical granule antigen recognized by the monoclonal antibody 1223 has also been observed following fertilization (Decker *et al.*, 1988). Extracellular enzyme activity has also been associated with morphogenesis in sea urchin embryos (Rapraeger and Epel, 1981), and a proteolytic enzyme has also been identified in blastocoelic fluid (Pointer, 1978). Further investigation of the precursors and products of the Sp12 antigen modification could provide more insight into the cortical granule - ECM transition which attends sea urchin fertilization.

The appearance of the 140K antigen between 24 hr and 48 hr is coincident with mesenchyme cell surface immunoreactivity. This correlation is strong evidence

that the 140K antigen is mesenchyme specific. It is of note that the gastrula and prism have proportionally more spicule and mesenchyme immunoreactivity than the pluteus, and the 140K band, prominent in the 48 hr and 84 hr samples but faded in the pluteus, is also prominent in the spicule enriched adult test extract. There is a possibility that this prominent antigen corresponds to msp130, the 130K primary mesenchyme antigen identified by Leaf *et al.* (1987), Anstrom *et al.* (1987) and previously characterized with the monoclonal antibody 1223 (Carson *et al.*, 1985; Farach *et al.*, 1987). Measurements of the M_r of the Sp12 band have consistently yielded a value greater than 150K, however, the effect of different SDS-PAGE buffers and parameters, used in different laboratories, could give a disparity in M_r of this magnitude. Detailed comparisons of the western blot patterns provided by the three antibodies in question (done in one lab) could help resolve this question.

The transition from non-feeding prism to feeding pluteus is accompanied by another molecular transition of the Sp12 antigens. The 120K band in the gastrula (48 hr) is resolved into two bands at 120K and 105K in the pluteus (120 hr). The broadening of the band and eventual resolution into a doublet implies molecular processing of the antigen. This change is accompanied by fading of the 140K antigen as mentioned above. Older plutei retain the same pattern of bands as the 120 hr larvae, confirming that these changes are specific to the embryonic - larval transition and are maintained through larval development. Correlation of the events detected by western blot with those observed microscopically is not compelling evidence of a connection in this situation. Many events are occurring during this period (digestive system maturation, onset of feeding, pigment production, growth of larval arms, establishment of blastocoelar cell networks, etc.) and deciding which one (if any) is related to the change in antigen M_r is not possible.

The transformation into an adult results in some changes to the antigen profile, but one worth noting is the reemergence of the 140K antigen in the adult test. As noted above the prominence of this band in stages which contain a proportionally larger amount of spiculogenic cells coincides with its prominence in the exclusively spiculogenic tissue of the adult test. This correlation may reflect involvement of the 140K antigen in spiculogenesis in both embryos and adults.

The changes chronicled above imply that the Sp12 antigens are labile through early development. This lability is confused by the number of antigens recognized by Sp12, but it basically consists of two types: the appearance or disappearance of antigen(s) at specific embryonic stages; and the apparent modification of continuously expressed antigens. The *de novo* appearance of antigens in development is commonly seen in sea urchin embryos, but modification of antigens present through development has not been described in this embryo. Other systems, have well documented examples of such processing. The gene for N-CAM, neural cell adhesion molecule of the chick and other vertebrates, has alternate splicing patterns yielding three different polypeptides, and each of these can be differentially glycosylated so that a wide range of molecular weight variants can result from the single gene. Expression of the variants is spatially and temporally restricted through development (Edelman, 1986). Other examples of temporally regulated changes in M_r include antibody class switching (see Honjo *et al.*, 1981 for review), and keratin gene modulation during skin differentiation (Fuchs and Green, 1980). The appearance of the 105K antigen could result from functional modulations of the 120K antigen, which are reflected in a change in its M_r (as in N-CAM). Another possibility is proteolytic processing of the 120K antigen as the larva matures - similar to the explanation of the shift in banding pattern seen at fertilization. It has been shown that a yolk glycoprotein complex is proteolytically processed into a number of discrete bands through blastulation (Lee *et al.*, 1989).

Proteolytic events may actually be essential for sea urchin morphogenesis. Inhibition of a metalloendoprotease blocks *in vitro* spiculogenesis (Roe *et al.*, 1989).

Whether the changing pattern of the Sp12 antigen bands through development is related to their function is not clear. Nor is the mechanism used to generate the pattern apparent, although proteolytic processing is one possibility. These antigens will require examination in much greater detail before any conclusions to these questions can be reached.

Multiple antigens

Three potential sources for the large number of immunoreactive bands in the immunoprecipitations are: Sp 12 is not monoclonal, but consists of multiple antibodies of different specificities; artifactual proteolysis of a large protein precursor has yielded several immunoreactive fragments; or, Sp12 recognizes a redundant epitope present on a number of antigens.

The possibility that Sp12 is not monoclonal is highly unlikely, given the number of antibodies required to give this range of specificities, the cloning results, the stability of the cell line, and the presence of a single immunoglobulin subclass.

Artifactual proteolysis is also highly unlikely. It was controlled for by the inclusion of protease inhibitors for each of the four classes of proteinase (Darrett, 1980), by carrying out the procedures at reduced temperatures, and by restricting storage of extracts. In addition, direct evidence that proteolysis does not contribute to the number of antigens observed is provided experimentally (Fig. 14).

The results presented here provide good evidence that Sp12 recognizes a redundant epitope. Identification of the epitope's chemical nature was done by enzymatic and physico-chemical degradation with a variety of reagents. The results from this approach must be interpreted with caution because of potential ambiguities in the behaviour of peptide and carbohydrate moieties. The epitope's resistance to enzymatic digestion by chitinase, hyaluronidase, and ribonuclease is

not surprising given the antigens' behaviour in PAGE, and supports the view that these antigens are glycoproteins. Proteolytic enzyme degradation of immunoreactivity has been interpreted as evidence of a peptide epitope (Trimmer *et al.*, 1987), however, this may simply reflect release of an undigested sidechain containing the epitope. Amido black staining of protease digested samples run on PAGE indicate that the epitope is lost rather than degraded. Furthermore, the maintenance of Sp12 immunoreactivity following detergent, heat, and 7M guanidine denaturation are not consistent with a protein epitope. In contrast, the epitope is partially degraded by periodic acid, is completely eliminated by endoglycosidase F digestion, and remains immunoreactive when held at 100°C (neutral pH) for up to 60 min. Endoglycosidase F and the potential contaminant PGNase both specifically cleave N-linked oligosaccharides (complex or polymannose) from their core peptides. These results indicate that the Sp12 epitope is on an N-linked carbohydrate moiety.

N-linked carbohydrate moieties can be generally classified as either polymannose oligosaccharides, or complex oligosaccharides with chains terminating in - N-acetylglucosamine - galactose - sialic acid (Kornfeld and Kornfeld, 1980; Lennarz, 1983; 1986). It would be expected that a polymannose chain would be susceptible to periodate oxidation, and resultant destruction of epitopes (Spiro, 1966; Woodward, 1985). Complex oligosaccharides would be less affected since although sialic acid is rapidly oxidised by periodate, N-acetylglucosamine is not oxidised unless substituted at C6 (Spiro, 1966). It is still possible that Sp12 recognizes the core of a high mannose chain, but since epitopes usually reside at exposed sites (*i.e.* at the ends of carbohydrate sidechains) the hypothesis that a sialic acid residue is oxidized to reduce antibody binding without eliminating it, is more likely. Further evidence that Sp12 recognizes a complex oligosaccharide is the lack of correlation between con A labelling (which binds polymannose strongly) and

Sp12 labelling on western blots (Fig. 12). While definitive characterization of the putative carbohydrate epitope awaits inhibitor or sequencing studies, these observations provide preliminary evidence that Sp12 recognizes a complex oligosaccharide chain.

The developmental inhibitors utilized all work through disruption of specific molecular moieties. Proline analogs and β APN inhibit collagen production (Wessel and McClay, 1987), β -D-xylosides inhibit proteoglycan formation (Kinschita and Saiga, 1979), sulphate-free SW inhibits production of sulphated molecules (self-evident), and tunicamycin inhibits glycosylation of glycoproteins (Schneider *et al.*, 1978). While disruption of collagen production and proteoglycan formation inhibit gastrulation and further morphogenesis, the Sp12 antigens are still expressed on mesenchyme cell surfaces once they have ingressed into the blastocoel. This indicates that differentiation of Sp12 antigen expression is not necessarily coupled to normal morphogenesis. The caveat in these cases is that mesenchyme is still able to migrate within the blastocoel - this may be the minimum requirement for Sp12 antigen expression.

Sulphate-free SW and tunicamycin usually blocked Sp12 antigen immunoreactivity, but also inhibit mesenchyme migration more completely. This result could mean that Sp12 recognizes a sulphated carbohydrate epitope, or it could be an artifact indicating Sp12 antigen expression is dependent on previous mesenchyme migration or the previous expression of other glycoproteins and sulphated molecules. The isolated animal hemisphere immunoreactivity seen in the SFSW experiment argues for a migratory-permissive role of sulphate rather than a direct contribution to a sulphated epitope. Tunicamycin may play a more direct role in disrupting Sp12 immunoreactivity since the drug inhibits N-linked glycosylation and there is strong biochemical evidence that Sp12 labels an N-linked oligosaccharide. Tunicamycin is a toxic drug and the pattern of protein synthesis in

mesenchyme blastulae can be perturbed by it (Heifetz and Lennarz, 1979), although this is not a factor in the expression of msp130's protein core (Anstrom *et al.*, 1987). In summary, the enzymatic, oxidation, boiling, and inhibitor experiments all indicate that the Sp12 epitope is a complex, N-linked carbohydrate moiety. The issue of whether or not it is sulphated is not resolved because of the many effects sulphate deprivation has on development, and because some embryos grown in SFSW were able to express the Sp12 epitope.

Sp12's recognition of a post-translational modification provides a ready explanation of the epitope's redundancy. The question remains then, what is the relationship between these glycoproteins, all of which share the same carbohydrate sequence recognized by Sp12? Does this redundancy imply a common functional attribute for this sidechain? It would seem logical to assume that a shared molecular structure would share a common function, but this is yet to be established for the Sp12 epitope. Clearly, more detailed investigations of the isolated molecules are required to answer these questions, but initial classifications of the antigens (presented below) were undertaken on the basis of their membrane affinity and tissue specificity. The function of the epitope (and antigens) is investigated in Chapter 6.

Antigen membrane affinity

The *in situ* ontogeny of the Sp12 antigens revealed labelling of two discreet tissues: the hyaline layer in early embryos, and mesenchyme cell surfaces following their ingression. The segregation of antigens to both ECM and cell surfaces is confirmed by cell surface labelling of dissociated live cells, and by the presence of antigens in glycine extractable ECM.

Cell surface labelling in hatched blastulae is an unexpected finding of this analysis, however, it is consistent with the *in situ* data. The bilaminar, apical cell surface/hyaline layer margin, labelling apparent in fixed blastulae is confirmed by

the labelling of a presumably apical cap of antigen on dissociated live blastulae cells. This pattern of blastomere labelling has been observed with another monoclonal antibody (Ic10) and the antigen it recognizes is colocalized with cytoplasmic actin filaments (Nelson and McClay, 1988). A number of other apical markers retain their cellular polarity in dissociated cells, despite removal of apical ECM. These include the nucleus, and a specific class of vesicle (Nelson and McClay, 1988); microvilli and cortical pigment granules (Schroeder, 1988); and egg surface antigens recognized by polyclonal antibodies (Yazaki, 1984). The Sp12 antigens localized to blastomeres appear to be another such marker. These antigens do not change in localization despite being held with antibody bound for up to three hours - they do not cap. The actin colocalization seen by Nelson and McClay (1988) provides a mechanism of anchoring for that antigen, perhaps a similar mechanism is involved in anchoring Sp12 antigens.

Western blot analysis of these fractions established the tissue segregation of these antigens. There is a discreet separation of antigens; the ECM extract contains a complementary set of bands to that retained on membranes. The two populations of Sp12 antigens present in blastulae are a high M_r fraction which is in the hyaline layer matrix, and a few lower M_r components which remain intimately associated with the blastomere cell surfaces. The hyaline layer stratification alluded to in Chapter 3 reflects the separation of these two antigen populations, and their different M_r 's may indicate differences in their ability to interact with the plasma membrane.

Prism (72 hr PF) blastomeres showed less prominent caps of antigen, and in plutei (120 hr PF) there are only vestiges of this apical labelling. These live cell labelling results confirm the *in situ* observation that Sp12 labelling of the hyaline layer gradually decreases. While their blastodermal immunoreactivity decreases through time, the immunoreactivity of mesenchyme derivatives in these stages is

uniform, intense, and unaltered by incubation in antibody. Although these cells do not have a polarity like the blastodermal cells, the behaviour of their Sp12 cell-surface antigens is identical. This may reflect a common mechanism of antigen anchoring.

Western blots of these two stages' membrane and ECM antigens reflect the generalization that high M_r antigens segregate to the ECM and low M_r antigens segregate to the plasma membrane. However, the pluteus sample has a less discreet set of high M_r ECM bands, and several of them appear to be faint analogs to the more intense membrane bands. This may represent the loss of hyaline layer antigens (high M_r) (seen *in situ*) and the subsequent release of mesenchyme antigens. If there is Sp12 immunoreactive ECM in the blastocoel (as implied by the immunogold localization) it must be closely associated with cell surfaces since the *in situ* ontogeny does not reveal any grossly distinguishable non-cellular immunoreactivity. A possible model explaining the pattern of antigens on the 120 hr western blot could include mesenchyme antigens with a range of M_r s whose extractability is dependant on proximity to the membrane. Those in close proximity are bound, without specificity as to M_r , and there is a pool of these same antigens in the pericellular matrix which can be extracted with CMFSW or 1 M glycine. Of course, it may be that all of these antigens are bound to the membrane and their presence in the ECM extract reflects a normal distribution of all such molecules extractability with these solvents, *i.e.* this extraction is not an all-or-none phenomenon, but is graded and these "ECM" antigens are in the easily extracted part of the curve. Despite these unresolved questions, it is apparent that very high M_r antigens are associated with the hyaline layer, and lower M_r antigens are associated more strongly with plasma membranes.

The results of the membrane extractions are not easily interpreted, but they do indicate the general nature of the cell surface antigens' interactions with the

plasma membrane. Increased or decreased ionic strength does not release any amount of antigen, nor does non-ionic detergent at 4°C. Elevated pH, heated non-ionic detergent (Triton X-114), and ice cold ionic detergent do release antigen from the membrane preparations. The moderate pH elevation released a small amount of antigen; since a higher pH (11.0) is reported to release peripheral membrane molecules (Fujiki *et al.*, 1982), this may indicate Sp12 antigens are peripheral. However, this is not consistent with the Triton X-114 data, since nonionic detergents would not be expected to extract peripheral membrane molecules (Bordier, 1981). The protocol utilized required heating the preparation to 60°C to separate the aqueous and detergent phases in the Triton X-114 extraction. The degree of heating used may have increased the kinetic energy of the antigens enough to escape the ionic interactions holding them in place. Another argument for a peripheral association is the segregation of solubilized antigens to the aqueous phase of the Triton X-114 extraction buffer, behaviour that would not be expected of integral molecules with hydrophobic regions (Bordier, 1981).

The distinction between integral and peripheral membrane molecules has been examined in greatest detail in the red blood cell ghost, in which membrane proteins discreetly segregate into these two categories (Steck and Yu, 1973; Yu *et al.*, 1973; Steck, 1974). Despite the appeal of this relatively simple model, there is little reason to expect all cells have equally discreet membrane components. More complicated mechanisms of cytoskeleton anchoring and protein-protein interactions are possible (*e.g.* the talin and vinculin interactions with the fibronectin receptor), and such complexities could smear distinctions between integral and peripheral. Membrane bound Sp12 antigens do not have clear properties of either integral or peripheral components, however, they do appear to be anchored to cytoskeletal matrix through a robust non-covalent interaction. Anchorage to the cytoskeleton could explain the lack of Sp12 antigen capping as well as their behaviour in

peripheral/integral membrane protein solvents (Koch, 1981). A final caution must be raised, however, the Sp12 antigens are a heterogeneous group of glycoproteins - there may be integral *and* peripheral molecules among them, in which case an unambiguous characterization with Sp12 would be difficult.

Antigen cyto-specificity

The labelling of three mesenchymal cell types with this antibody provides a molecular link between them. While detailed analyses of the antigens expressed by each cell type have not been undertaken, skeletogenic mesenchyme cultures appear to express a similar number of antigens as whole embryo immunoprecipitates. In contrast, the separated endoderm/mesoderm and ectoderm fractions appear to contain very different components. This apparent contradiction may arise from the nature of the isolated components. The filter separated fractions are solubilized immediately after separation, and are washed in both 1 M glycine and in CMFSW - all soluble ECM should be removed, while the cultured mesenchyme had been allowed to grow and secrete antigens for four days. Another point of less significance is the purity of the two fractions. The filter separated fractions appear very pure, while the cultures have many non-spiculated cells contaminating them. The source and nature of these non-spiculated cells remains unknown.

Similarities in the mesodermal fraction and the culture indicate that the 185K and 120K antigens are restricted to mesenchyme. The large number of antigens on the ectodermal fraction must represent both the vestiges of apical blastodermal cell antigen apparent in the live cell labelling as well as the antigens on the intensely labelled pigment cells. While these are not easily separated, on the basis of labelling of both fixed and live ectodermal/pigment cells the pigment cell antigens must be the major component in this fraction. A salient point in identifying the pigment cell antigens is their origin. The rise in pigment cell immunoreactivity correlates very closely with the fall in blastodermal immunoreactivity. Given the

pigment cells' location, behaviour, and range of antigens it may be that they are scavenging antigen from blastodermal cells, and presenting it on their own surfaces. The numerous issues raised by cellular specificity of the antigens await further resolution, but it is interesting to note a discreet difference in the antigens on ectoderm and those on endoderm/mesoderm. This difference, once clarified, may provide further clues as to the function of the specific antigens.

The numerous biochemical investigations recounted in this chapter generally resulted in more questions than answers. This is the nature of such investigations. Resolution of many of these questions may be possible with more specific probes. Sp12 identifies an interesting group of glycoproteins, and their relationships to each other are highlighted by this antibody. However, identification of discreet functions and localizations of individual antigens requires a reductionist approach to this group of molecules. Immunological methods are one viable way of dissecting this group of antigens, using a panel of monoclonal antibodies derived from Sp12 purified immunogen. This pursuit is justified if individual antigens have localizations and/or functions which warrant further investigation. Chapter 5 provides information on a poorly described tissue that Sp12 antigens are localized to. Chapter 6 investigates the function of the Sp12 antigens, as a group, through antibody mediated steric disruption of epitopes and antigens. The results of such investigations should indicate whether the Sp12 antigens deserve further attention on an individual basis.

CHAPTER 5 MESENCHYME DERIVATIVES IN THE SEA URCHIN EMBRYO

INTRODUCTION

Mesenchyme has traditionally been divided into two classes in the sea urchin embryo, primary and secondary. This division is based on the time of ingression of these two populations of cells: primary ingressing before archenteron invagination, and secondary separating from the tip of the archenteron after secondary elongation starts (at about one quarter gastrula). Examination of a wide range of species and the production and use of lineage markers has shown that the timing of mesenchyme release is not as discreet as previously thought (Schroeder, 1981; Gibson and Burke, 1985; Wray and McClay, 1988). This finding has reduced the value of the primary/secondary label, and as a result, more descriptive terms based on the derivative cell type have arisen. In *S. purpuratus* the primary mesenchyme, more specifically skeletogenic mesenchyme, ingress at 28 - 34 hr PF, pigment cells or chromogenic mesenchyme ingress during archenteron invagination at 40 - 44 hr PF (Gibson and Burke, 1985), and classical secondary mesenchyme ingress during secondary elongation of the archenteron at 44 - 48 hr PF. Myogenic mesenchyme is released from the coeloms early in their development (72 - 84 hr PF), and give rise to the muscles of the esophagus (Burke and Alvarez, 1988).

As discussed in Chapter 1, skeletogenic mesenchyme has been investigated in great detail over the last two decades. Methods for culturing these cells *in vitro* have been worked out (Okazaki, 1975b; Harkey and Whiteley, 1980; Mintz *et al.*, 1981) as have techniques for manipulating these cells *in vivo* (Langelan and Whiteley, 1985; Ettensohn and McClay, 1988). In addition, a number of primary mesenchyme specific monoclonal antibodies (Carson *et al.*, 1985; Leaf *et al.*, 1987; Farach *et al.*, 1987; Anstrom *et al.*, 1987; Shimizu *et al.*, 1988) and nucleic acid probes (Harkey *et*

al., 1988, Wilt *et al.*, 1987) have been developed. Chromogenic mesenchyme and pigment cells have been investigated in some detail (Gibson and Burke, 1985; 1987; 1988; Gibson, 1987), as have the events of esophageal myogenesis (Ishimoda-Takagi *et al.*, 1984; Burke and Alvarez, 1988). However, the late ingressing secondary mesenchyme, which form the blastocoelar cells, has received little attention.

Blastocoelar cells play a prominent role in the morphogenesis of the primitive gut, exhibit complex migratory behaviour after they ingress, and take on a distinctive morphology and localization in the larva. In this chapter the ontogeny, morphology, localization, and behaviour of these cells are documented. From these observations tentative conclusions are drawn concerning the function of the blastocoelar mesenchyme.

MATERIALS AND METHODS

Immunofluorescence

Indirect immunofluorescence on a variety of embryonic and larval stages was undertaken as described in Chapter 3. Sp12 was used, both at low dilutions to document the positions and numbers of all three mesenchymal derivatives labelled, and at higher dilutions to examine the blastocoelar cell patterns in isolation. The shift in specificity of Sp12 at higher dilutions was described in Chapter 2. Other lineages based on indirect immunofluorescence were obtained from the literature.

Nomarski DIC and time-lapse observations

The ingression and migration of mesenchyme, and the behaviour of mesenchymal derivatives *in vivo* was observed using Nomarski differential interference contrast (DIC) optics. Gastrula stage embryos were deciliated with an osmotic shock. A small aliquot of pelleted embryos was suspended in 50 volumes of a 1:1 solution of FSW:DW, centrifuged (50g, 1 min) and resuspended in FSW. Embryos remained 100% viable after this treatment, but lost the cilia required for

swimming. Embryos were placed on a clean glass microscope slide, covered with a coverslip, and were held immobile for several hours with light compression. Although cilia regenerate within three to four hours, the initial compression was sufficient to hold embryos in place for up to 12 hours. Later stages, especially plutei, do not require deciliation and could be held in place with compression alone. Plutei were observed for up to two days using this system.

Specimens were held at 15 °C using a thermoelectric cooling stage (Cloney *et al.*, 1970). This temperature was sufficient to allow normal development over the period embryos were viewed.

Time-lapse video microscopy utilized a Panasonic low-light surveillance camera and Panasonic time lapse video recorder, coupled to the Nomarski microscope. Still photomicrographs were taken with an Olympus 35mm camera using Plus-X film.

Electron microscopy

Specimens for scanning electron microscopy were fixed in 4% glutaraldehyde, 2% osmium tetroxide in 0.45 M sodium acetate buffer at pH 6.5 (21 °C, 1 hr). They were dehydrated through a graded ethanol series then critical point dried in carbon dioxide. Specimens were fractured following the method of Morrill (1986). Briefly, dried specimens were adhered to a stub using sticky tape, then a slide covered with sticky tape was lowered onto their exposed surface. The slide was lifted off, fracturing the specimens and exposing the blastocoel and its contents. Stubs were then sputter coated with gold and viewed in a JEOL 300 scanning electron microscope.

RESULTS

Mesenchyme derivatives in the sea urchin embryo

The four known fates of mesenchyme cells in the *Strongylocentrotus purpuratus* embryo (Fig. 24) are outlined above, and some details are provided in Chapter 1. The following is a brief overview to put the blastocoelar cell results in context. Primary mesenchyme are the first to ingress, and they give rise to the skeletogenic cells. Following this ingress there is a period of relative stability in the vegetal plate and the mesenchyme migrate over the blastodermal basal lamina. Eventually the cells coalesce in their typical circumferential pattern around the vegetal pole. In gastrulae, ventrolateral clusters of cells have formed, and after fusion of their fine pseudopodia, each cluster secretes a calcareous, triradiate spicule within its syncytial cytoplasm (Okazaki, 1975a; Gustafson and Wolpert, 1967). The spicules elongate in all three directions to form the characteristic larval skeleton.

The chromogenic mesenchyme, which give rise to the pigment cells ingress just at the onset of primary invagination of the archenteron, and migrate up the blastocoel wall. Between gastrula and prism they penetrate the blastodermal basal lamina, re-enter the epidermis, and start to synthesize echinochrome pigment (Gibson and Burke, 1985; 1988). Pigment cells remain motile throughout larval development and although they are usually well spaced in the ectoderm, aggregations occur at the arm tips and apex of the larva. Occasionally pigment cells are seen in close apposition to blastocoelar cell processes.

During gastrulation, cells at the tip of the archenteron extend long pseudopodia which contact the blastodermal basal lamina in the animal half of the embryo. These pseudopods guide and pull the archenteron during the second phase of its elongation (Dan and Okazaki, 1956; Hardin, 1988). As the archenteron extends across the blastocoel, the pseudopodal cells are released from its tip. The

mesenchyme released at this time retain a fusiform shape, and eventually take up positions on both the blastoderm and the archenteron. They become multipolar with several points of attachment to the blastoderm, the archenteron, and the developing skeleton. These blastocoelar cells (Chapter 3), appear to retain their points of attachment, and their multipolar shape through the rest of larval development.

Myogenic cells give rise to the esophageal muscles of the larva and are released from the coelomic epithelium at late prism. They migrate a short distance onto the esophageal basal lamina (Burke and Alvarez, 1988) and differentiate contractile filaments (Ishimoda-Takagi *et al.*, 1984; Burke and Alvarez, 1988). Functional muscle bands are present in the early pluteus (96 hr PF).

The potential role of larval mesenchyme in adult skeletogenesis was discussed in Chapter 3. It is not clear whether calcoblasts are derivatives of larval mesenchyme (Fig. 24), or are spontaneously differentiated late in development. Coelomic cells are not discussed here, since the coeloms are epithelial outgrowths of the archenteron rather than condensations of freely migrating mesenchyme cells (Gustafson and Wolpert, 1963; Burke and Alvarez, 1988).

Of the four mesenchymal cell types identified in *S. purpuratus*, the blastocoelar cells are the most obscure. Their labelling by Sp12, involvement in gastrulation, and unique morphology prompted a detailed examination of them.

Blastocoelar cells

Nomarski DIC optics reveals many details about blastocoelar cell shape and location (Fig. 25). They are multipolar with extensive pseudopodia spanning long distances in the embryo. These cell processes connect the skeletal rods to the gut and epidermis of the larva (Fig. 25b,c), and extend around the gut, along skeletal rods and adjacent to the epidermal basal lamina. They form a scaffolding or lattice work in the early pluteus that is attenuated but maintained in older larvae. In some

regions of the larva, the processes appear to merge and redivide establishing a reticulum of pseudopodia (Fig. 25a). Blastocoelar cells are concentrated around the anus and intestine (Fig. 25c), in the larval arms adjacent to the skeletal rods, and dorsal to the esophagus. They occur less densely along the skeletal rods (Fig. 25b), around the stomach, ventral to the esophagus, and adjacent to the ciliary band (Fig. 25a), especially in the dorsal and ventral transverse portions. Cell bodies are also suspended in the blastocoelar space by processes extending to the gut, epidermis, and skeletal rods.

Indirect immunofluorescence using Sp12 delineates more clearly the extent and location of blastocoelar cell processes (Fig. 26). High dilutions of Sp12 reduces skeletal rod and skeletogenic mesenchyme fluorescence (Chapter 2) allowing clearer visualization of blastocoelar cells and their interrelationships (Fig. 26a). Fusiform cells extend along the skeletal rods occasionally sending processes to the gut or epidermis. A tangle of processes around the intestine and extending to both skeletal rods is also apparent. Sp12 at lower dilutions shows blastocoelar cells in relation to pigment cells and skeletogenic cells (Fig. 26b,c). Cells on the dorsal side of the esophagus and stomach send processes out to the epidermis where they appear to contact pigment cells (Fig. 26b). The nature of this contact is not clear. On the ventral surface, circumanal cells appear to do the same thing (Fig. 26c). Relationships between blastocoelar cells are also apparent in these preparations (Fig. 26b, c).

High magnification views of labelled cells show the different shapes of blastocoelar and skeletogenic cells on skeletal rods (Fig. 27a, b). The degree of reticulation in blastocoelar cell processes is also clear at high magnification (Fig. 27b, c). Numerous large branching pseudopodia are a distinctive trait of all blastocoelar cells observed. This complexity varies between cells, those on skeletal

rods have fewer and simpler pseudopodia (Fig. 27a) while those suspended in the blastocoel have extensively branched processes (Fig. 27c).

Optical sections of whole mount specimens provide a two dimensional view of these cells, but it is difficult to appreciate their three dimensionality. An artist's impression of the blastocoelar cell distribution was constructed from high dilution immunofluorescence images (Fig. 28). This drawing effectively portrays the architecture of the blastocoelar cell processes, and their role in connecting different regions of the embryo.

Blastocoelar cell ontogeny

Blastocoelar cells form a complex network of cell bodies and processes in the early pluteus, but little is known of their development. The ontogeny of Sp12 antigens presented in Chapter 3 suggests that these cells are secondary mesenchyme derivatives; the events leading from gastrula stage mesenchyme to blastocoelar cell networks in the pluteus are documented further here.

Time-lapse video provides a continuous view of the detachment of secondary mesenchyme from the tip of the archenteron, and their subsequent migrations (Fig. 29a-f). Initially the cells extend thin pseudopodia to anchor to the ectodermal basal lamina, and after establishing contact, cell bodies are released from the archenteron epithelium. Secondary mesenchyme migrations are not extensive but are characterized by short advances of the cell, pseudopodial extension and anchoring, and repositioning of the cell body. In this way one cell establishes a number of pseudopodial contacts on the ectoderm, the archenteron, and the developing spicules (Fig. 29d). The cell body occupies a position between the contacts, either suspended in the blastocoel or on the basal lamina of the ectoderm or endoderm. Once established, the cells appear to be permanently localized; in embryos observed for up to 10 hours, no blastocoelar cell changed location after establishing its pseudopodial contacts. While direct observation of the ontogeny of these cells in

pluteus arms were not undertaken, their morphology and antigen expression combined with descriptions of arm development (Gustafson and Wolpert, 1961) are congruent with their development from secondary mesenchyme.

Investigations using still photomicrographs illustrate the blastocoelar cell ontogeny with greater resolution (Fig. 30). Although several different specimens are examined, these preparations have the advantage of showing changes in blastocoelar cells over several days of development. Secondary mesenchyme released from the archenteron during the last half of gastrulation retain a fusiform shape and establish pseudopodial contacts with the ectoderm and archenteron (Fig. 30a). By prism stage, 24 hours later, cells associated with the gut have extended long, broad processes around the gut and to adjacent skeletal rods and epidermis (Fig. 30b). Five day plutei also have blastocoelar cells with numerous processes, but as the larvae enlarge the pseudopodia became attenuated (Fig. 30d). There is not a dramatic increase in the number of cells to fill the enlarged volume of the larva, and cells appear smaller and more dispersed. Blastocoelar cells in older plutei (10 days PF) are much like those of five day plutei (Fig. 30f). The cell bodies are small in comparison to the volume they occupy, and pseudopodia are long and attenuated. The localization of blastocoelar cells does not change; they are concentrated in the arms, along the ciliary band, around the gut and along the skeletal rods, with occasional cell bodies suspended in the blastocoelar space (Fig. 30b-f).

To quantify the contribution of blastocoelar cells to the developing embryo, cell counts were made at various stages in development. These were done in two ways: using Sp12 as a fluorescent marker of all mesenchyme, and assigning the blastocoelar fraction on the basis of cell shape; and counting the number of secondary mesenchyme cells (again using shape and location as indicators) visible with standard light microscopy (Fig. 31). The numbers of cells from each of the

methods coincide at about the prism stage (72 hr PF) when blastocoelar cells are fully immunoreactive.

Twenty embryos were counted for each stage, and results are presented as the mean for one embryo plus or minus one standard deviation. At full gastrula (48 hr PF) 3.9 ± 3.0 cells are Sp12 immunoreactive, while 13.2 ± 2.3 fusiform cells are present in the blastocoel. By prism (72 hr PF) 21.4 ± 2.8 cells are labelled by Sp12, while 20.0 ± 2.3 are visible in the blastocoel using light microscopy. In four arm plutei (120 hr PF) 22.4 ± 3.5 cells are immunoreactive and 25.2 ± 3.2 are identified by light microscopy. Older plutei (7 days PF) have 28.8 ± 3.4 labelled cells (Fig. 31).

Blastocoelar cell fine structure and behaviour

Greater resolution of blastocoelar cells and their environment is achieved with the use of scanning electron microscopy (Fig. 32). At half gastrula many cells at the tip of the archenteron are loosened from their epithelium, poised for release (Fig. 32a). Cells already released span the blastocoel between the archenteron and blastoderm. Little branching of pseudopodia is apparent and the blastocoel is relatively free of fibres or other evidence of ECM. More cells are visible in full gastrulae (50 hr PF), and there is an increased amount of fibrous material in the blastocoel (Fig. 32b). At early prism (60 hr PF) coelomic pouches are visible and blastocoelar cells are evenly distributed over the differentiating gut (Fig. 32c). Although this specimen has no cells suspended in the blastocoel, processes do extend from cells on the gut to the larval skeleton. Late prisms (80 hr PF) have fibrous ECM adjacent to the gut and epidermis (Fig. 32d). Some blastocoelar cells on the gut are flat while others retain their fusiform shape, however, all of these cells have extensive pseudopodia. Cell processes divide to fine strands, coalesce into broad pseudopodia, and interconnect cells extensively. Processes form a reticulum around the intestine, and link the gut to the epidermis and skeleton (Fig. 32d).

Cells released from the archenteron between half gastrula and full gastrula have a simple, fusiform shape (Fig. 32e). Pseudopodia extend to the blastoderm, archenteron, and skeletogenic clusters. These cells look similar to mesenchyme in bipinnaria larvae of the starfish *Pisaster ochraceus* (Chia, 1977). In contrast to sea urchin larvae, mesenchyme in bipinnaria do not develop into elaborate multipolar blastocoelar cells and they remain motile throughout larval development (B. Crawford, pers. comm.). *S. purpuratus* prism cells are multipolar (Fig. 32f), and fine fibres become a prominent component around them. Cellular processes elongate and branch as the embryo grows. Pseudopodia taper to a fine point in early larvae (Fig. 32e), but mature into a variety of contacts in prisms. Broad termini on adjacent cells, fine insertions into underlying epithelia, and indistinguishable connections to neighbouring cell's pseudopodia may all be utilized by a single cell (Fig. 32f).

The shape, localization and ontogeny of blastocoelar cells is documented above. This characterization provides information on what these cells are and where they come from, but gives little indication of why they are there. To better establish the function of these cells in the embryo, their ultrastructure and behaviour were examined.

Blastocoelar cell ultrastructure was briefly examined during immunogold localization of Sp12 antigens in 48 hour and 72 hour embryos (Chapter 3). The fixation required for immunogold processing is not sufficient to allow detailed subcellular examination, however, some characteristics are clearly apparent. These cells contain numerous vesicles of different densities, and Golgi bodies are prominent even though fixation is less than optimal. They appear to be secretory as Sp12 antigen is contained within the Golgi and vesicles.

Behaviour of the blastocoelar cells was examined using time lapse video and timed still photographs (Fig. 33, 34). Early pluteus (96 hr PF) blastocoelar cell

bodies, suspended between the ectoderm, gut, and skeletal rods, are not maintained in a static position by the cell processes. Cell bodies show dramatic translocations along the cytoplasmic processes (Fig. 33). This behaviour appears to be an extension of the process these cells use to establish their pseudopodial contacts earlier in their ontogeny. The pseudopodia of cells with translocating cell bodies do not detach, and multipolar contacts are maintained. In early plutei large vesicles are also translocated along cytoplasmic strands (Fig. 33c).

Blastocoelar cell bodies are translocated in older plutei as well, in a more accentuated manner than in early plutei (Fig. 34). Time-lapse video recordings reveal pronounced activity along the cytoplasmic strands of blastocoelar cell processes, with vesicles and cell bodies rapidly transported from one strand into another across great distances. These vesicular and cell body translocations are most clearly seen in cells suspended in the blastocoelar space. Movements within these cells are not unidirectional, and net movements preserve the established scaffold of cytoplasm.

Figure 24. The fate of gastrula stage mesenchyme cells in the pluteus larva. Four mesenchymal derivatives have been identified in the *Strongylocentrotus purpuratus* larva. Primary mesenchyme (first ingressing, 30 hr PF) become skeletogenic cells; chromogenic mesenchyme (which ingress just at the start of archenteron invagination, 40 hr PF) migrate into the epidermis and become pigment cells; blastocoelar mesenchyme (released from the archenteron tip during gastrulation, 44 - 48 hr PF) interconnect embryonic tissues as blastocoelar cells; and myogenic mesenchyme (released from the forming coeloms, 72 hr PF) which migrate to the esophagus and form the esophageal muscles. It is not clear whether skeletogenic mesenchyme or blastocoelar mesenchyme (or both) derivatives secrete the adult calcareous structures. Indeed, calcoblasts may arise from newly differentiated cells rather than from either of these lineages. Drawings are not to scale.

24

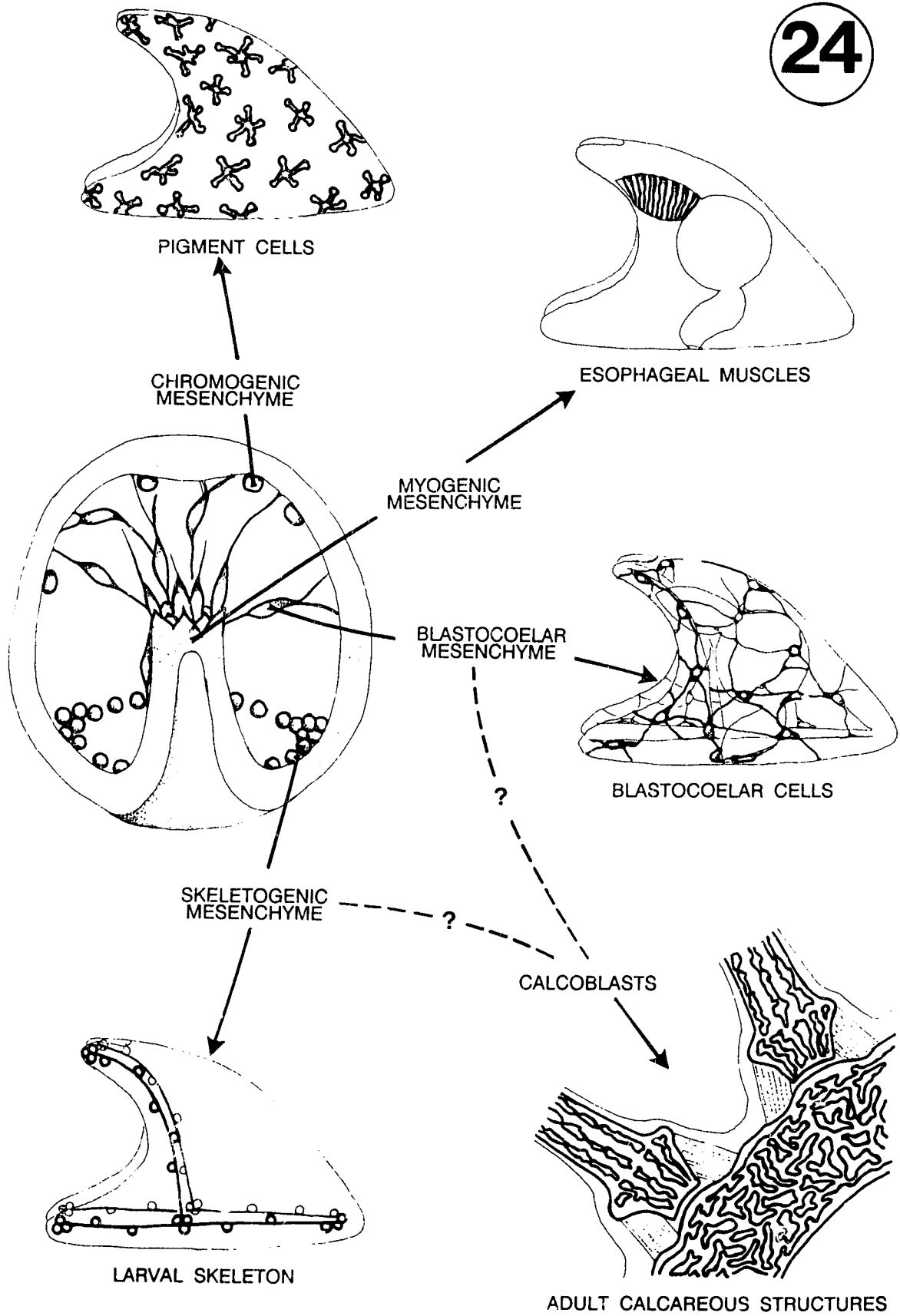


Figure 25. Nomarski interference contrast micrographs of blastocoelar cells in the 5 d pluteus. **a)** Dorsal view of the oral hood region. This optical section is just below the epidermis. Four cell bodies (arrowheads) and numerous interconnecting cytoplasmic strands are visible. At least one cell is always associated with the ciliary band (cb) in this region. Another is associated with the left anterolateral skeletal rod. **b)** Ventral view of the left body rod of the skeleton in the gut region. Fusiform cells on the skeletal rod are blastocoelar cells (arrowhead) extending processes to the stomach (st) and epidermis (arrows). **c)** Ventral view of the subepidermal region around the anus (a). Two cells (arrowheads) are closely applied to the intestine and extend processes to adjacent cells, skeletal rods, and epidermis. Scale bar is 20 μm .

Figure 26. Sp12 immunofluorescence micrographs of blastocoelar cells. **a)** Sp12 diluted out to reduce primary mesenchyme labelling. Blastocoelar cells are on the skeletal rods, around the gut, in the oral hood, and anterior to the gut. Cells have a typical shape and extend numerous processes. **b)** Dorsal optical section, just under the epidermis, of a pluteus labelled with Sp12 at lower dilution. Blastocoelar cells form an extensive network of processes that appear to contact the pigment cells (arrows). **c)** Ventral optical section of the same larva. The cells form a network of processes around the gut, and again appear to interact with pigment cells (arrows). Scale bar is 20 μm .

Figure 27. High magnification views of immunolabelled blastocoelar cells. **a)** Blastocoelar cell (arrowhead) on the skeletal rod extends processes along the rod and to the gut. **b)** Blastocoelar cell (arrowhead) near the apex of the embryo. The processes are highly reticulated and connect to the skeletal rod and epidermis. **c)** Complex tangle of processes around the stomach of a pluteus. Blastocoelar cells (arrowhead) are connected to each other, skeletal rods, and the gut. Scale bar is 10 μm .

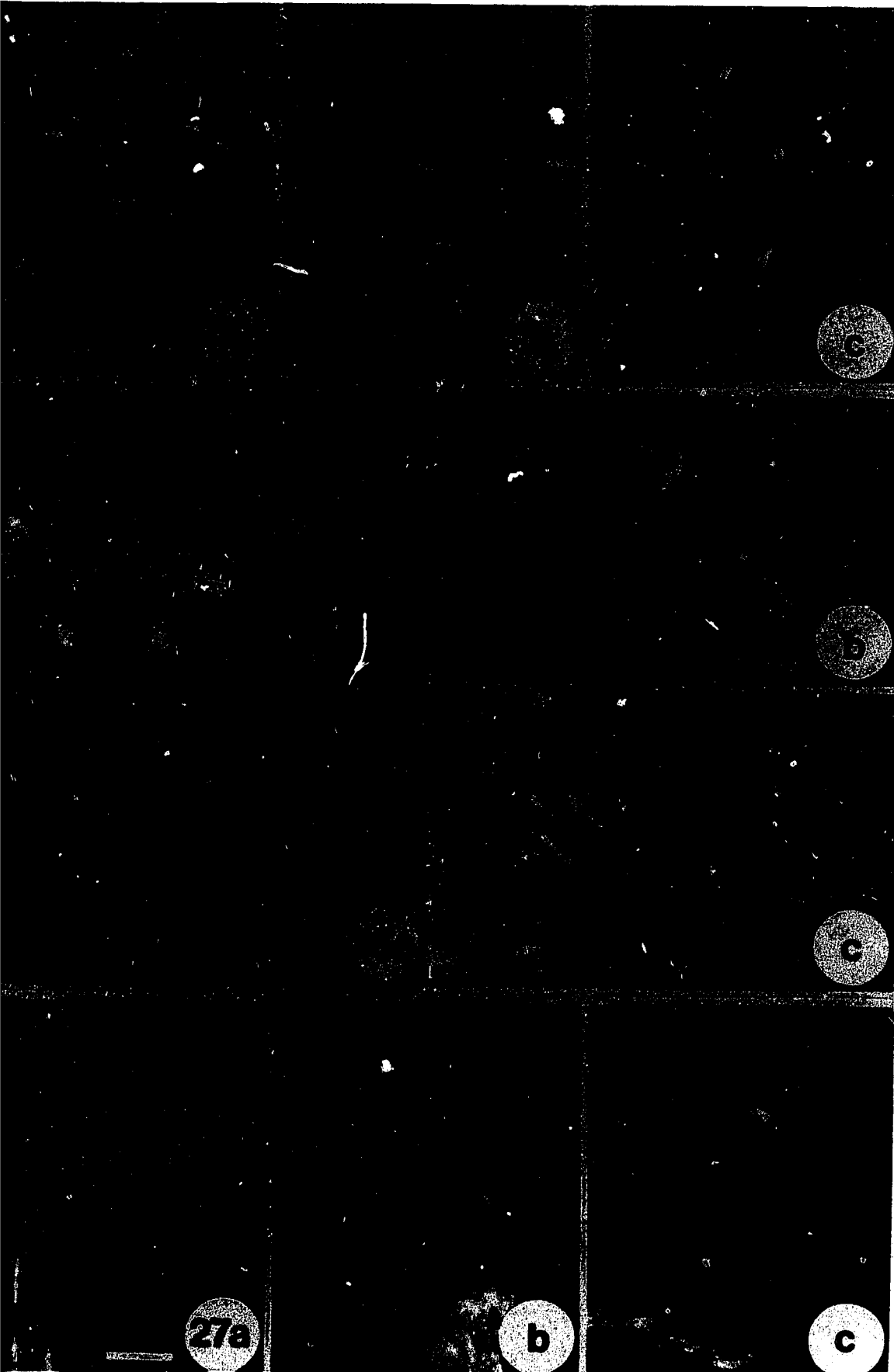
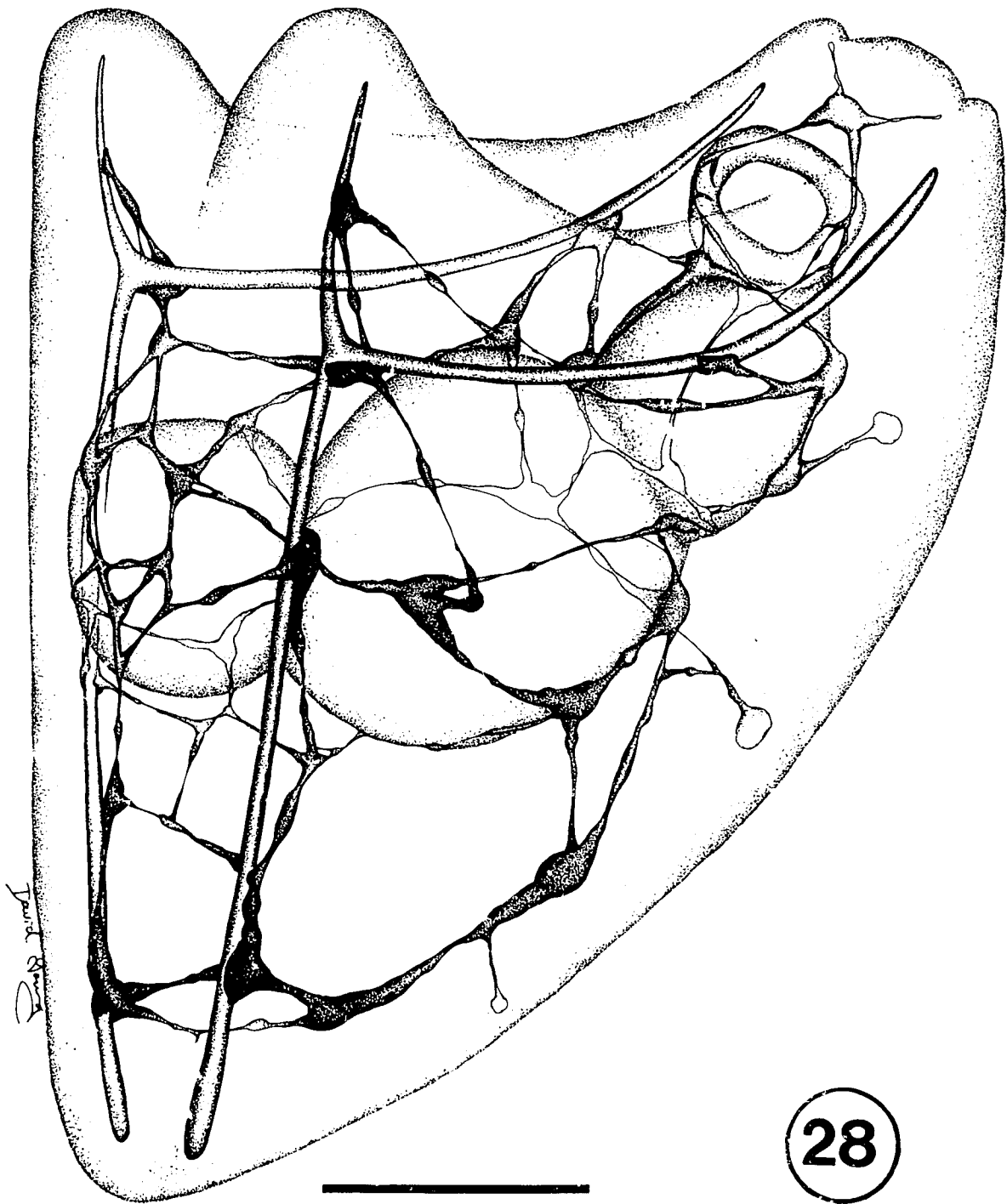


Figure 28. Artist's three dimensional representation of the blastocoelar network in a 96 hr early pluteus. This ventrolateral view reveals most of the blastocoelar cell network. Cells are extensively interconnected, and are primarily aggregated around the gut. Cells are also situated on the skeletal rods and processes extend to the epidermis, and ciliary bands. The precise site of cell bodies is variable, but the general plan of the scaffolding their processes form is well conserved. Details of pseudopod attachment to the basal lamina are not known, although the connections are insensitive to CMFSW disruption. Vesicles within processes are actively translocated, even cell bodies are moved back and forth along thin cell processes. Despite this, the cells are anchored by their processes, and do not migrate after their initial localization. As the coeloms are flattened sacs on the lateral surface of the esophagus, they are poorly delineated and are not detailed in this representation. Scale bar is 50 μm .



28

Figure 29. Time lapse video sequence of gastrulation. Examination of a single blastocoelar cell (arrowhead) and its processes (arrows) through several hours of gastrulation, photographed at 50 min intervals. **a)** After release from the archenteron tip this fusiform cell extends down along the blastodermal basal lamina. **b)** The cell appears to be closely applied to the blastoderm, migrating vegetally. It is bipolar and processes extend to both the animal and vegetal regions. **c)** While two fine processes still connect it to the vegetal region, the cell has retracted to its animal pole attachment, and taken on a rounder shape. **d)** The cell has extended again and appears tripolar. **e)** The two vegetal contacts are prominent, a pseudopod extends to the skeletogenic mesenchyme cluster and a branch off it attaches to the blastoderm. The animal hemisphere attachment remains intact. **f)** The cell body is retracted to the animal hemisphere again and two pseudopodia are seen connecting it to the archenteron. Scale bar is 20 μm .

Figure 30. Nomarski differential interference contrast micrographs of blastocoelar cell development. Six different specimens from three-quarter gastrula to 10 d pluteus illustrate blastocoelar cells (arrowheads) through development. **a)** Soon after leaving the archenteron tip fine filopodia connect these cells to the blastodermal basal lamina, the archenteron, and the forming spicule. **b)** Blastocoelar cells are found in the same location in early prisms, associated with the gut, the blastoderm, and the spicules. **c)** In the late prism blastocoelar cells are associated with the same tissues. **d)** In the early pluteus, cells remain associated with the skeleton and gut. **e)** There is not a great increase in the number of cells in the 5 d pluteus, although processes are more extensive and branched (arrows). **f)** Two cell bodies and numerous cell processes are apparent (arrows) in this 10d pluteus. The number of blastocoelar cells and their locations appear similar to e), but there is elaboration of the cell processes. Scale bar is 20 μm .

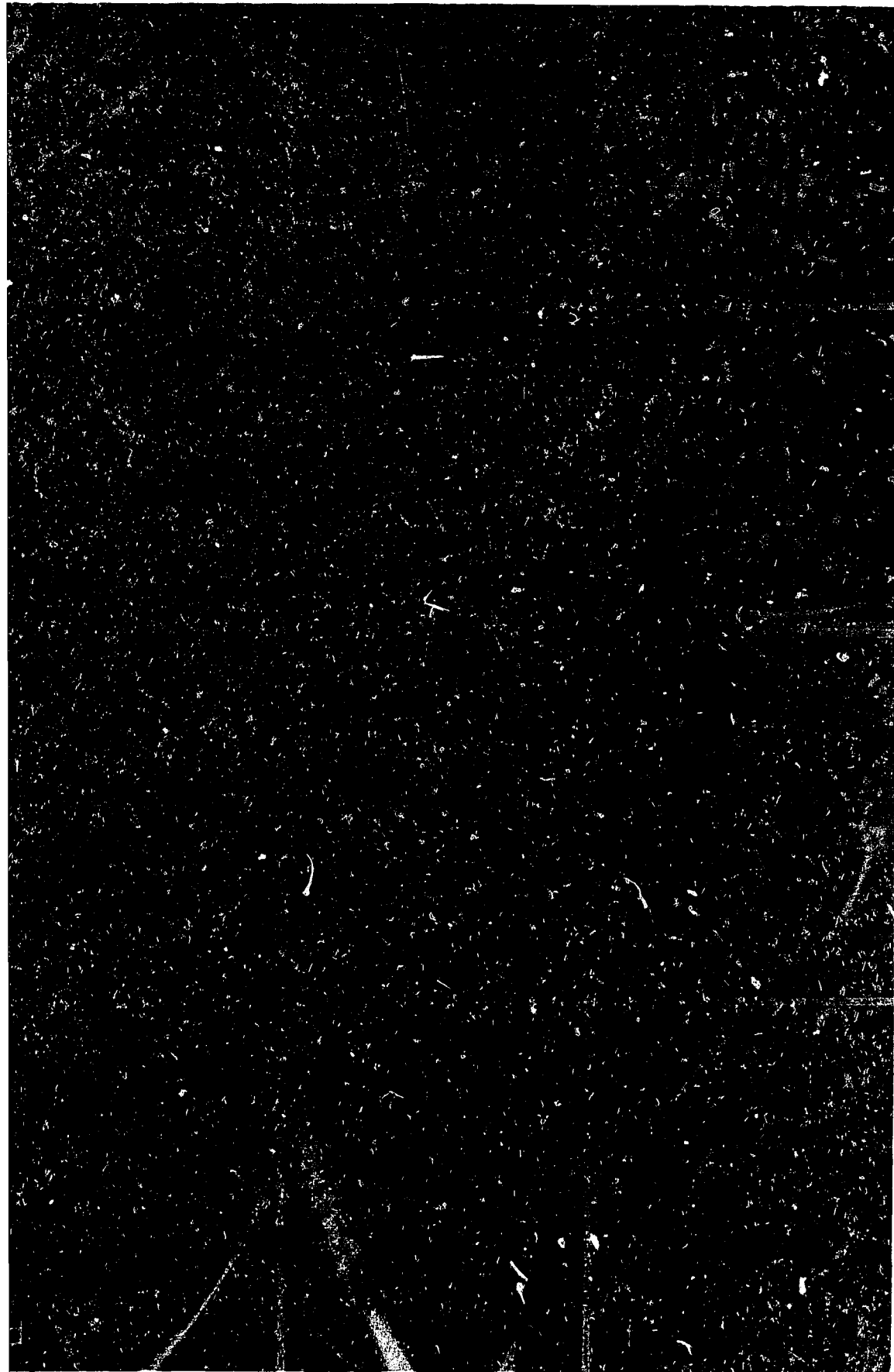


Figure 31. Graph showing number of blastocoelar cells versus hours PF. Both Sp12 immunoreactive cells and cells identified solely on the basis of morphology are plotted. Both counts are zero at 42 hr, as blastocoelar mesenchyme is not released until after secondary elongation has started. Points are means of 20 individuals and bars represent plus or minus one standard deviation. There is a dramatic rise in number from the start of gastrulation through to prism, but the increase appears to flatten somewhat in the pluteus. Immunofluorescence micrographs indicate that the number of blastocoelar cells does not increase proportionally to the increase in other tissues during pluteus growth. However, further data is required to confirm this numerically.

31

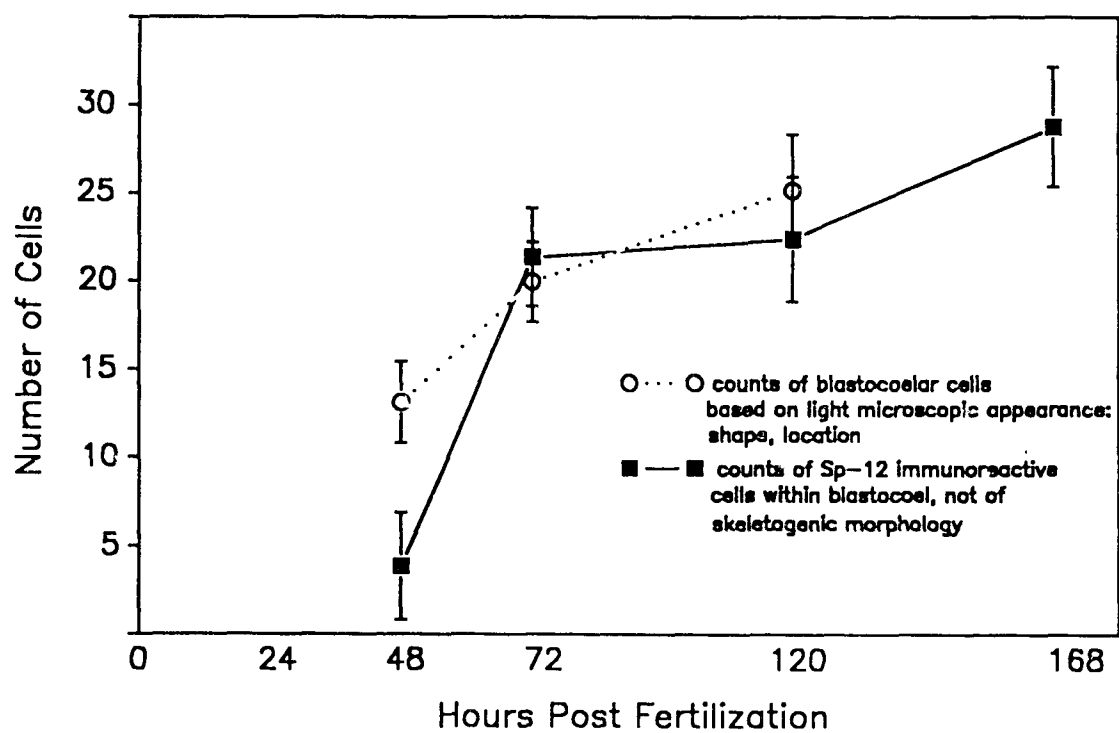
NUMBER OF BLASTOCOELAR CELLS THROUGH
EARLY DEVELOPMENT

Figure 32. Scanning electron micrographs of the blastocoel. These micrographs of specimens from half gastrula to prism show blastocoelar cells with much greater resolution than light micrographs. **a)** Half-gastrula. Skeletogenic mesenchyme (round cells in the vegetal blastocoel; arrows) have formed a ring around the invaginating archenteron. Cells at the archenteron tip have loosened and appear ready to migrate out. One blastocoelar cell appears to have already spanned the blastocoel between the archenteron and blastoderm (arrowhead). **b)** Postero-dorsal view of a full gastrula. Numerous fine processes extend from the forming coeloms (arrowhead), and blastocoelar cells are prominent in the blastocoel. Cells are associated with the gut, the blastoderm, and spanning the two (arrows). Extensive networks of cell processes are apparent. **c)** Posterior view of an early prism. Coeloms are further developed, and blastocoelar cells are densely placed on the primitive gut. Few blastocoel spanning cells are evident, although there are cells on the blastoderm (arrows). **d)** Early pluteus. Cells are flattened and more extensively branched in older larvae. They are embedded in an extensive fibrillar ECM. Cells clasp the gut and span the blastocoelar space (arrows). Processes are complex and reticulated (arrowhead). **e)** Blastocoelar cell soon after separating from the archenteron tip. Although it is still fusiform, at least four pseudopodia (arrows) extend to the archenteron (a), blastoderm (bd), and primary mesenchyme. Little ECM is apparent in the blastocoel at this point. **f)** Blastocoelar cell of an early prism, on basal lamina adjacent to the blastopore (future anus). This cell is clearly multipolar, and is flattened onto the basal lamina. It extends at least six pseudopodia which are further branched or fuse to adjacent cells (arrows). A fibrillar ECM is more apparent at this stage, and becomes increasingly prominent as the larva develops. Scale bar is 20 μm in a - d, and 5 μm in e and f.

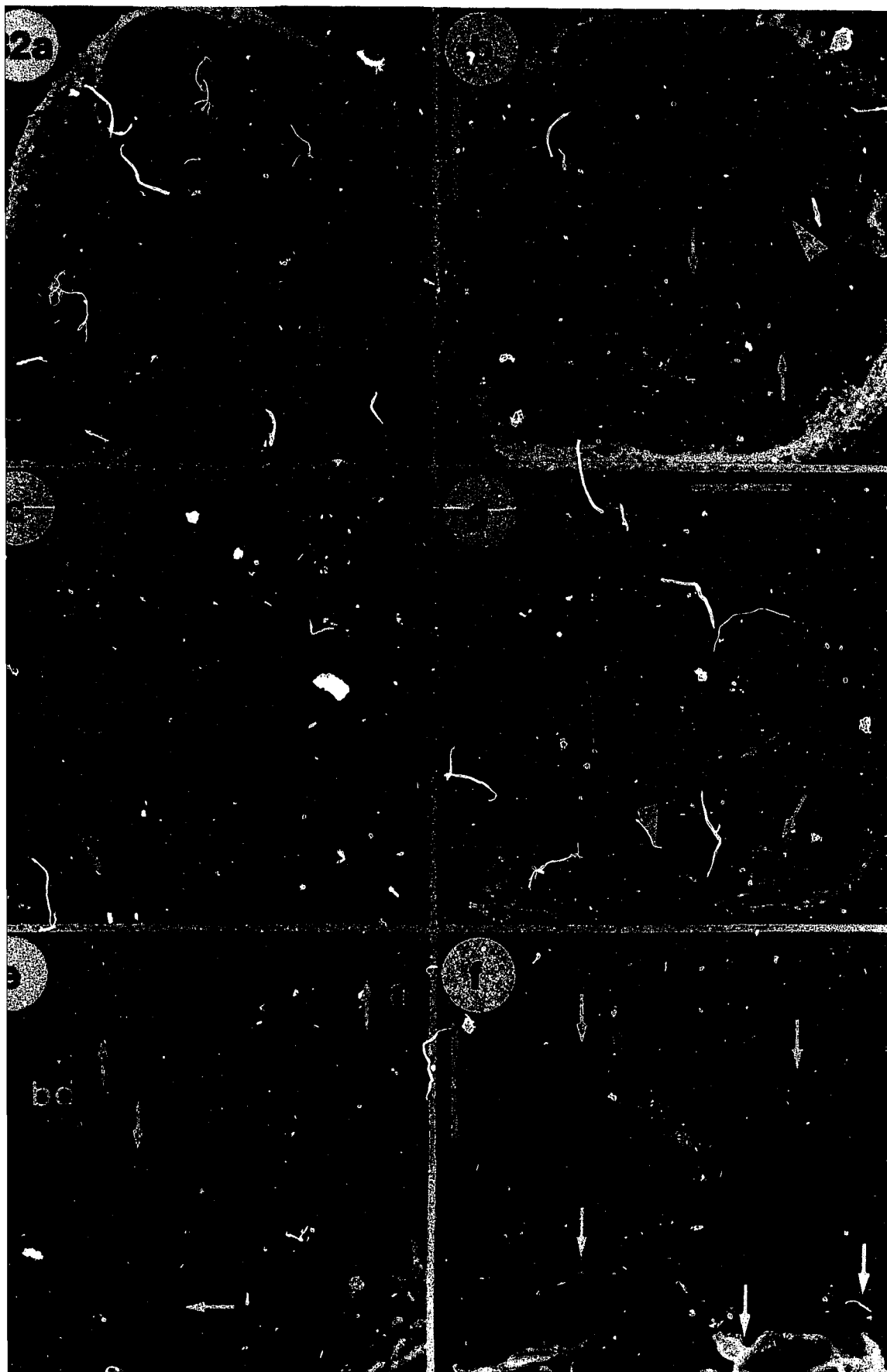


Figure 33. Blastocoelar cell activity in the 96 hr early pluteus. Sequential still micrographs taken at 12 min intervals with Nomarski DIC optics. The cell body of this blastocoelar cell was shifted across the posterodorsal blastocoel in 25 min. Pseudopodial connections to the dorsal epidermis and stomach are not released, but become attenuated during the translocation. The movements of several large vesicles in these and other pseudopodia are also apparent in this sequence. **a)** The cell body (arrow) is suspended in the blastocoel, adjacent to the epidermis. **b)** 12 min later the cell body (arrow) is closer to the stomach than the epidermis. A fine, branched pseudopod still extends to the epidermis. **c)** 12 min later, the largest part of the cell body (presumably enclosing the nucleus) is adjacent to the stomach (arrow). The branched pseudopod is still extended to the epidermis, and contains several vesicles (arrowheads). Scale bar is 10 μm .

Figure 34. Blastocoelar cell activity in a 7 d pluteus, monitored with timelapse video microscopy. This region of the embryo is ventrolateral to the stomach, just posterior to the ventral ciliary band. **a)** The round cell body (arrow) is suspended in the blastocoelar space by at least four thin pseudopodia which extend to the ciliary band, stomach, and adjacent skeletal rod. **b)** 10 min later the cell body has shifted back, up, and toward the stomach. As in the 96 hr pluteus, the pseudopodia do not detach, but become attenuated. **c)** 10 min later the cell has moved further back and up and is entirely out of focus. Swellings in some of the pseudopodia indicate rapidly translocated vesicles (arrowheads). Scale bar is 10 μm .



DISCUSSION

Sea urchin mesenchyme

Mesenchymal cells in vertebrates are defined as "small stellate cells, 7 - 10 μm in diameter, with several extensions of variable length, held in contact by means of nexus with those of adjacent mesenchyme . . ." (Krstic, 1984). These cells are not highly differentiated and are surrounded by an amorphous ground substance. They are prominent in embryonic tissues and are considered stem cells which give rise to a host of connective tissue and muscle derivatives. This description has to be modified to apply to sea urchin mesenchyme. As indicated above all freely migrating cells (non-epithelial cells) in the sea urchin embryo are considered mesenchyme. Furthermore, they are still considered mesenchyme after they have differentiated (*e.g.* skeletogenic mesenchyme). They are however, stellate cells with processes of variable length (and diameter) which interconnect adjacent cells, and they are of mesodermal origin. While homology with vertebrate mesenchyme can be elaborated, it is more fruitful to simply define sea urchin mesenchyme. Review of a number of comprehensive descriptive papers (MacBride, 1903; Gustafson and Wolpert, 1967; Okazaki, 1975a) indicates that sea urchin mesenchyme is defined as cells that have ingressed into the blastocoel, and then migrate as single cells, *i.e.* they are mesodermal and non-epithelial. Most mesenchyme differentiates soon after ingressing, so little more can be said to define them without danger of excluding one or another cell type.

The descriptions provided in this chapter have focussed on one mesenchyme derivative, the blastocoelar cells. Comparisons of these cells with similar vertebrate cells has resulted in a hypothesis of their function.

Blastocoelar cells

The filiform network derived from classical secondary mesenchyme has been noted in several descriptive accounts of sea urchin development (Macbride, 1903;

Gustafson and Wolpert, 1967; Okazaki, 1975a). The three dimensional scaffolding produced by the blastocoelar cell processes is strikingly similar to the arrangement of vertebrate mesenchymal cells, or the further differentiated fibroblasts in loose connective tissue proper (Krstic, 1984). Their mesenchymal origin is consistent with this analogy. The evidence of their secretion of Sp12 antigens provided in Chapter 3 is also consistent with the fibroblast analogy. A generalized secretory role would not be unexpected given their location and the consistency of the blastocoelar "gel" ground substance which surrounds these cells (Strathman, 1989).

It is not known if the complex and highly interconnected blastocoelar cell processes share cytoplasm, forming a syncytium, or if the cells simply join in adnexa. In *plutei* stripped of their ectoderm, blastocoelar cell processes retain their contacts with the basal lamina (data not shown). This indicates an interaction with the epithelial basal lamina rather than with the epithelial cell membranes as postulated by Gustafson (1963), but does not exclude more intimate autologous contacts between blastocoelar cells. Syncytial contacts would be conducive to a physiologic function of this tissue while discreet cells in apposition would support a structural role. Resolution of this issue awaits further studies.

Gustafson (1969) and Ryberg (1977) considered these cells a nerve plexus on the basis of their shape and their positive reaction with catecholamine detecting stains. However, Burke showed in *S. purpuratus* (1978b) and in *Dendraster excentricus* (1983) that these cells do not have any features of nerve cells, while another population of cells show ultrastructural and histochemical characteristics of nerves. The blastocoelar cells' mesenchymal antigens and the ontogeny presented here indicate that they are of mesodermal origin, while neurons are typically of ectodermal origin. Further repudiation of the neuron theory is provided by Bisgrove and Burke (1986; 1987). Their detailed studies with neurotransmitter

specific antisera do not show any spatial or temporal correlation of neuron development with that of blastocoelar cells.

Ryberg and Lundgren (1977) described cytoplasmic strands underlying the ciliary band and considered them neurons. While histochemically localized neurons are present in the region of the ciliary band they are not apparent in the light microscope (B. Bisgrove, pers. comm.), but blastocoelar cell processes are prominent in this region. Similarly, Gustafson's observation of filiform neurons delaminating from the ciliated band ectoderm (Gustafson and Toneby, 1971) may simply have been misinterpretation of cell bodies being actively translocated from the ectodermal basal lamina out into the blastocoel. As noted above, these cells are very active and cell bodies are continually being shifted from one filopodial strand into another.

There is little evidence to support the suggestion that these cells are neuronal, they appear to be a pool of connective tissue cells with a low degree of determination. Burke (1978b) postulated that they are undifferentiated mesenchyme utilized in larval growth and metamorphosis. While they appear to be partially differentiated (they are actively secretory), their fate does remain labile. They are easily redirected into alternate differentiation states.

It has been shown that the late ingressing mesenchyme can replace missing skeletogenic cells (Langelan and Whiteley, 1985; Ettensohn and McClay, 1988), an ability consistent with Burke's interpretation. However, this need would only rarely (if ever) arise in nature, and these cells represent a prominent component of the blastocoelar space of embryos and larvae. While they may represent "spare part" cells involved in elaboration of adult structures later in larval life or during metamorphosis, the evidence presented above indicates they have a connective tissue role in early larvae. The analogy with fibroblasts is relevant again as their

differentiation is also somewhat plastic - they are known to transform into adipose cells (Krstic, 1984).

The broad specificity of Sp12 does indicate parallel patterns of differentiation in the labelled cell types. They are obviously related through their mesenchymal designation, essentially a behavioral classification, however, their shared expression of Sp12 antigens indicates a complementary molecular relatedness. Whether this sharing of antigens reflects a common functional attribute or is some higher order of lineage marker (*i.e.* all migratory cells express this epitope) is a moot point, but intuitively the epitope could be expected to have a function required by all three cell types.

The behavioral traits blastocoelar cells exhibit are difficult to interpret, but the prominent translocation of vesicles and cell bodies indicates that these are metabolically active cells. Perhaps the fibroblast analogy can be extended into the realm of blastocoelar cell mediated tractional structuring (Stopak and Harris, 1982) as one of the processes involved in pluteus morphogenesis. This would be consistent with their precursors' role in extending the archenteron in gastrulation (see Chapter 1; Hardin, 1988). Ultrastructural evidence supports this possibility, secondary mesenchyme cells contain numerous microtubules, while filopodia are full of microfilaments (Tilney and Gibbins, 1969). In competent larvae smooth muscle components have been identified in these cells (Cameron and Hinegardner, 1978). This possibility could be explored using blastocoelar cell cultures on silicone rubber coated coverslips (Harris *et al.*, 1980).

The numbers of cells in embryos and larvae increase in an expected manner given their abrupt generation during gastrulation and sparse distribution in older larvae. Their potential contribution to adult structures late in larval existence or during metamorphosis awaits more detailed examinations of this period of

development. Similarly, resolution of their intercellular connectivity requires further investigation.

In summary, the blastocoelar cells arise from secondary mesenchyme following a brief period of migration within the blastocoel. They extend numerous fine pseudopodia and form a network of cytoplasmic processes around the gut, along the skeletal rods, within the larval arms, and suspended within the blastocoel. They are very active cells, translocating vesicles and cell bodies along the pseudopodia, and appear to be secretory. Their shape, ontogeny, location, ultrastructure, and behaviour are analogous to those of fibroblasts in vertebrates. Correlation of these characteristics is not proof of these cells' role in development, but it provides a hypothesis for testing in future studies. Examination of blastocoelar cells may further illuminate the processes of morphogenesis and differentiation.

CHAPTER 6 FUNCTION OF THE SP12 ANTIGENS

INTRODUCTION

The hypothetical basis of this project is that cell surface molecules, especially those which have a developmental expression restricted in time and space, are potentially involved in morphogenesis. Antibodies specific to such molecules are useful reagents, not only for the identification and characterization of the antigens they label, but also for perturbation of these antigens' function. Perturbation experiments have been employed in a number of different developmental systems, with results that include disrupted migration, pattern formation, intracellular events initiated by receptor-ligand binding (as at fertilization), and differentiation. Disruption of sea urchin development has utilized anti-ECM and anti-cell surface reagents, including both monoclonal and purified polyclonal antisera. While these experiments are outlined in Chapter 1, it is useful to summarize them here.

Anti-ECM solutions against laminin causes blastodermal deformation (McCarthy and Burger, 1987). *In vivo* labelling of a fibronectin-binding acid polysaccharide causes multiple or deformed spicule formation (Iwata and Nakano, 1985a), while *in vitro* this same antibody enhances spicule formation and calcium uptake (Iwata and Nakano, 1985b). Another monoclonal antibody which labels microfibrils in the blastocoel appears to delay secondary mesenchyme migration and dispersion (Burke and Tamboline, 1990). Anti-hyaline layer antibodies against both hyalin (Adelson and Humphreys, 1988), and apical lamina components (Myers, 1990) reversibly block gastrulation and further morphogenesis. There are fewer examples of anti-cell surface antibodies perturbing morphogenesis. The anti-Meso 1 monoclonal antibody appears to block mesenchyme migration, but this has not been well documented (McClay *et al.*, 1987; McClay and Etensohn, 1987a). The monoclonal 1223, which labels msp130, blocks spiculogenesis *in vitro*, but *in vivo*

results have not been published (Carson *et al.*, 1985). Examination of the 1223 epitope reveals that it is an N-linked oligosaccharide which binds calcium (Farach-Carson *et al.*, 1989). The authors conclude that this cell surface carbohydrate moiety binds and sequesters calcium before it is internalized and incorporated into the larval skeleton. The anti-pigment cell antibody Sp1 has no effect on pigment cell migration or morphology *in vitro*, or when whole larvae are incubated in SW containing it (Gibson, 1987).

From the above, it is clear that this technique can provide significant information regarding the labelled antigen's role in development *if* labelling of live cells has an effect. Not all antibodies do have an effect, but the promise of positive results make such an analysis desirable. This chapter documents *in vitro* and *in vivo* experiments using Sp12 as a perturbing agent during development.

MATERIALS AND METHODS

Whole embryo incubations

To assess the effect of Sp12 on developing embryos, cultures were incubated in FSW with antibody. Eggs were fertilized in FSW containing 1 mM aminotriazole (Showman and Foerder, 1979) and Sp12 ascites diluted 1:500, 1:1000, and 1:1500. Controls were incubated in NRH ascites at the same concentrations. After fertilization the cells were washed three times in FSW, then cultured in 150 μ l of FSW (with antibody added) at a density of 1000 embryos/ml. Embryos and larvae were examined and photographed at 24 hour intervals, and aliquots were fixed for immunofluorescent probing with secondary antibodies.

This method does not expose the mesenchyme or its derivatives to antibody, so two techniques were employed to overcome this problem. Mesenchyme were brought into the external milieu by dissociation and *in vitro* cell culture; and

antibody was introduced into the blastocoel - the mesenchymal environment - by microinjection.

In vitro analysis

Hatched blastulae and full gastrulae were dissociated to a single cell suspension as outlined in Chapter 4. The cells were pelleted (1000g, 10 min), resuspended in MFSW with penicillin (0.3 units/ml) and streptomycin (0.5 μ g/ml), and counted in a hemocytometer. Glass cover slips were cleaned in acid alcohol and placed in sterile 30 mm plastic petri dishes. Three ml of cell suspension (5×10^6 cells/ml) was placed in each petri dish and cells allowed to settle on the coverslip. After 6 to 12 hours (14°C) coverslips were washed by gentle pipetting and aspiration of overlying MFSW or by dipping coverslips in a beaker of MFSW. Under these conditions adherent cells are predominantly mesenchyme - skeletogenic mesenchyme in the blastula dissociates and skeletogenic, chromogenic, and blastocoelar mesenchyme in the gastrula dissociates.

Cells were initially plated and subsequently cultured (14°C) in MFSW with a variety of additional components. Sp12 and NRH ascites fluids were added at a dilution of 1:200 (approximate final antibody concentration of 30 μ g/ml). Protein A purified Sp12 was added to cultures at concentrations of 15, 30, 45, 60, and 75 μ g/ml; purified NRH was used at 20 and 65 μ g/ml. Sp12 Fab fragments were added to cultures at a concentration of 17.5 μ g/ml. Some cells were cultured in MFSW with 2% horse serum, which allowed differentiation of the primary mesenchyme and the growth of spicules.

After three to four days in culture, cells were fixed *in situ* by flooding the petri dishes with 4% paraformaldehyde in FSW (21°C, 1 hr). Coverslips were then rinsed thrice in FSW, and incubated in antibody solutions for immunofluorescent observation. Cells cultured in antibody were probed with secondary antibody after fixation to determine the degree of live cell labelling by antibody in the culture

medium. As NRH does not bind to the cells in culture they were labelled with Sp12 after fixation, followed by secondary antibody to provide detailed visualization of cell morphology. Fixed cells were probed with other antibodies as well: Sp14 which labels a blastocoelar ECM component; and Sp30 which is primary mesenchyme specific.

Labelled coverslips were mounted by inverting them onto a bath of mounting medium in a vaseline well. After washing the exposed side of the coverslip in DW, cells were viewed and photographed as outlined in Chapter 3.

In vivo analysis

To examine the effect of Sp12 *in vivo*, antibody was introduced into live embryos by microinjection. Injecting pipettes were pulled on a DKI vertical pipette puller, and backfilled with Sp12 and NRH ascites fluid or purified antibody in FSW. They were attached to a Picospritzer II injecting apparatus and clamped into a micromanipulator. A suction pipette attached to a screw operated air piston was clamped in a paired micromanipulator. The injecting chamber (a 30 mm petri dish filled with FSW) and pipettes were viewed through an inverted microscope (Bausch and Lomb) at 100X to 250X magnification. Embryos were delivered to the suction pipette with a small diameter pasteur pipette controlled by mouth pressure. After immobilization on the suction pipette the injecting pipette was inserted into the blastocoel of the specimen and antibody was delivered with microsecond pulses of nitrogen from the Picospritzer II. As the outlet diameter of the injecting pipette was an uncontrollable variable, the pressure and duration of the nitrogen pulses was varied to deliver an approximately equal amount of antibody solution into each embryo injected. The amount of solution injected was judged by the degree of embryo inflation during injection, and blastocoel volume was approximately doubled from $5 \times 10^5 \mu\text{m}^3$ to $10^6 \mu\text{m}^3$. After injecting, embryos were removed

with the mouth pipette and cultured in FSW in a 24-well plate (Falcon) at 5 to 10 embryos/well.

Embryos injected ranged in age from hatched blastula through to half gastrula. At each session, 5 to 20 individuals were injected with each antibody. After culturing for three to four days embryos were viewed on the Nomarski microscope or fixed in 4% paraformaldehyde and examined using indirect immunofluorescence.

RESULTS

Whole embryo incubations

Sp12 has no effect on fertilization, elevation of the vitelline layer, or secretion of the hyaline layer. Subsequent development through cleavage, blastula formation, mesenchyme ingression, and gastrulation are similarly unaffected (Fig. 35a, b). Up to full gastrula, FSW incubated embryos are identical to the Sp12 incubated and NRH incubated embryos at all three dilutions examined.

By prism the 1:500 dilution of both NRH and Sp12 have a toxic effect on embryos, and most are dead by 96 hours. The 1:1000 and 1:1500 dilutions are not toxic, and at early pluteus no differences are apparent between FSW, NRH and Sp12 incubated specimens (Fig. 35c, d). While NRH and Sp12 specimens remained indistinguishable, differences between them and FSW incubated controls are obvious in 120 hour plutei. There are anomalies in the epidermis and gut of the antibody incubated animals. Epidermal cells show some blebbing, and occasionally loose cells are observed on the outside of the animal. The gut is often collapsed, with a very small lumen and thick wall in the stomach and intestine. There is little difference between the two antibodies, although the NRH incubated animals appear to be more seriously afflicted.

Early plutei which have been incubated in antibodies at 1:1000 dilution were fixed and probed with secondary antibody (Fig. 35e, f). The secondary antibody binds to the ciliary band and gut of both Sp12 incubated and NRH incubated specimens. There is no apparent difference in either the amount or distribution of this immunofluorescence. Secondary antibody does not bind to this extent or with this localization when early plutei (96 hr PF) cultured in FSW alone are probed.

In vitro analysis

Antibodies can not cross the blastoderm or epidermal barrier, so when whole animals are incubated in antibody-FSW the antibody does not have access to the mesenchyme within the blastocoel. To assess antibody effects on mesenchyme cells, embryos were dissociated at the hatched blastula and full gastrula stages, the cells were cultured with antibodies *in vitro*, and cellular anomalies were documented. As with the immunofluorescent labelling, a degree of variability is evident in these experiments. The concentration of active antibody, the condition of the dissociated embryos, and the quality of the MFSW used as a culture medium, are all variables which can affect the quality of cell cultures. Despite some fluctuation in the quality of cultures, consistent trends are evident throughout the results of these experiments.

When cultured in MFSW/pen/strep adherent cells from dissociated hatched blastulae form extensive aggregates with numerous large pseudopodia interconnecting them. The NRH incubated cells are almost identical to MFSW incubated cells, hence only comparisons between NRH and Sp12 are made in most experiments. Typically, after three days incubation in NRH ascites (at 1:200), cells have formed large networks of branching pseudopodia (Fig. 36a, c). In contrast, Sp12 incubated cells (ascites, 1:200) occur as tight doublets or single cells, at much lower densities on the slips, and with fine pencil-lead filopodia extending out in a corona around the cell body (Fig. 36b, d). Coverslips fixed at daily intervals, starting

immediately after washing the slips, show progressively more pronounced differences between Sp12 and NRH.

To determine if cells are sensitive to the antibody effect after adhesion to the slip and washing, cells were plated out, washed after 24 hours, and then incubated in antibody solutions (ascites, 1:200) for a further three days (Fig. 36e, f). Although Sp12's effect is not as extreme as in continuously incubated samples, when added after 24 hours it still affects the morphology of the plated cells. Thick pseudopodia characteristic of NRH incubated cells are absent, and although there is evidence of lamellipodia on some Sp12 incubated cells, they are less prominent than in NRH cells. Sp12 incubated cells have the characteristic pencil-lead filopodia and are primarily single cells, with little of the intercellular contact typical of NRH incubated cells.

Analysis of the effect on mesenchyme cells *in vitro* was based primarily on cell morphology. To supplement this analysis a second, independent measure of cell function, secretion of ECM, was utilized to examine differences in Sp12 and NRH incubated cells. The monoclonal antibody Sp14 binds to a microfibrillar ECM component in the blastocoel of sea urchin embryos from early blastula through to pluteus (Burke and Tamboline, 1990). Primary mesenchyme cells secrete Sp14 reactive material when they are plated out *in vitro*. This material is apparent in four day MFSW cultures as wispy microfibrils overlaying the cells. It localizes over the cell processes and outlines the cell bodies. The secretion of Sp14 microfibrils is unaffected by incubation in NRH (ascites, 1:200), and their appearance is identical to MFSW controls (Fig. 36g). Sp12 incubated cells, however, show no evidence of Sp14 labelled material around the cell or overlying the corona of filopodia (Fig. 36h). The secondary antibody used to detect Sp14 also binds to antibody fixed onto the cell after culture in antibody solutions. Thus the Sp12 incubated cells are brightly fluorescent, while the NRH incubated cells (which do not have antibody

bound to the cell surface) are not. This cellular fluorescence makes detection of the Sp14 microfibrils more difficult, but careful examination reveals no evidence of Sp14 positive material around Sp12 treated cells.

The above results were obtained using crude ascites fluid diluted 1:200. To obtain a more accurate idea of the potency of the antibody effect, and to eliminate any potential contribution from other ascites components, purified antibody was utilized in a dose-response experiment. While titres of active antibody from initial purifications were too low to be detected with indirect immunofluorescence, a subtle effect on dissociated cells could be discerned (Fig. 37). Antibody purified from ascites on a Sepharose-Protein A column has a much higher titre, and produced more pronounced effects. Cells incubated in Sp12 at 75 $\mu\text{g/ml}$ and 60 $\mu\text{g/ml}$ have the same morphology and degree of intercellular connection as those incubated in crude ascites (Fig. 38c). This morphology is recognizable through the series to 15 $\mu\text{g/ml}$, but there is an increase in filopodial length, degree of filopodial branching, and number of cells in a cluster as the antibody concentration drops (Fig. 38a, b). Although the density of filopodia does not increase as the antibody concentration drops, at 15 $\mu\text{g/ml}$ the cells are closer in morphology to Sp12 ascites incubated cells than to NRH ascites incubated cells. The threshold of Sp12's effect has not been determined.

Gastrula dissociates were also utilized in *in vitro* assays of the Sp12 effect on mesenchyme. Dissociation of gastrula stage embryos and subsequent plating and washing results in a more heterogeneous array of adherent cells. They include skeletogenic mesenchyme, secondary mesenchyme (pigment cells and blastocoelar cells), and epidermal cells. Because skeletogenic and blastocoelar cells take on similar morphologies when plated without horse serum, it is not possible to distinguish between them in the gastrula dissociates. Despite this, it is clear that Sp12 has an effect on mesenchyme from gastrula dissociates similar to that seen in

blastula dissociates (Fig. 39). NRH incubated cells have the usual pseudopodia connecting them with other cells in their vicinity (Fig 39a). Sp12 incubated cells have more lamellopodial processes, but the pencil-lead filopodia in a corona-like arrangement are similar to those of blastula dissociates. This effect is seen in gastrula cells incubated in ascites at 1:200 and in those incubated in purified antibody at 75 $\mu\text{g/ml}$ (Fig. 39b). Epidermal cells create mats on these cover slips, or take on slug-like shapes, and have a highly vesicular cytoplasm. These cells are not Sp12 immunoreactive, a strong indication that they are epidermal, not mesenchymal.

A significant finding in the gastrula dissociates is the effect of Sp12 Fab fragments on these cells. Sp12 Fab at a concentration of 17.5 $\mu\text{g/ml}$ has no effect on the dissociated cells. These cells have extensive thick pseudopodia connecting them to adjacent cells, with no hint of the morphology associated with incubation in divalent Sp12 (Fig. 39c).

In vivo analysis

While divalent Sp12 has a clear effect on dissociated mesenchyme cells *in vitro*, these experiments do not address the effect of the antibody on these cells, or development in general, *in vivo*. To assess this, antibody was microinjected into the mesenchymal environment, the blastocoel. Six separate sessions of microinjection were undertaken, using antibody solutions ranging from crude ascites to ion exchange purified antibody. Stages between hatched blastula and half gastrula were injected, using 5 to 20 embryos in each experiment. Again, variability played a role in the results obtained, however, Sp12 injected animals were always compared to NRH injected animals from the same session to determine the specificity of the effect.

In five of the six sessions no difference was observed between the Sp12 injected and the NRH injected animals. Depending on the antibody solution used,

either the embryos developed normally and appeared identical to uninjected controls (Fig. 40a, b), or both Sp12 and NRH injected animals are grossly abnormal in their development (Fig. 40c-d). Only one experiment showed a difference in Sp12 and NRH injected embryos. In that case 4/12 Sp12 injected embryos were abnormal compared to 6/6 normal NRH injected animals.

While completely undifferentiated balls of cells are occasionally produced, the majority of the abnormalities observed involve the skeleton and its development (Fig. 40c, d). The most common effect is disruption of the shape and orientation of the skeletal rods. Infrequently, embryos with more than two triradiate spicules or foci of calcification are observed. Another common effect of the injections is an increase in the blastocoelar volume, and displacement of the archenteron to one side of the embryo (Fig. 40e, f). Blastocoelar cells are not affected by the injected antibody, they take up positions around the blastocoel and develop their characteristic multipolar morphology. Pigment cells are also unaffected, they migrate to the larval epidermis and differentiate pigment - even in embryos which are otherwise grossly abnormal.

The stage embryos are at during injection affects their subsequent development. Embryos are resilient to injections before gastrulation has started: animals injected from hatched blastula through to late mesenchyme blastula continue to develop, at least to the early pluteus stage. Embryos injected after gastrulation has started, once the archenteron has started to invaginate, are all dead within two days of injection.

Figure 35. Effect of incubating live embryos in Sp12. Embryos a, c, and e were incubated in Sp12 ascites diluted 1:1000 in FSW, from fertilization on. Embryos b, d, and f were incubated in NRH ascites diluted 1:1000 in FSW, from fertilization on. **a, b)** At one half gastrula there is no difference between the two treatments. Embryos in both cultures form normal blastulae, have normal primary mesenchyme ingression, and show normal gastrulation. The hyaline layer is not disrupted, nor is mesenchyme migration or morphology affected. The distorted appearance of the Sp12 incubated specimen is due to squashing during the preparation for examination. **c, d)** The early plutei also show no differences in hyaline layer or mesenchyme morphology. Pigment cells (c) have migrated into the epidermis, blastocoelar cells (b) have taken up their normal positions and shape, and spicules (sp) are absolutely normal. Again, differences in larval dimensions are due to squashing of the Sp12 specimen, in addition to the slight variation normally seen in larval size. **e, f)** 96 hr plutei fixed after incubation in Sp12 and NRH, then probed with secondary antibody alone. This shows the sites of primary antibody localization during live embryo incubations. Sp12 is not bound to any of the structures it labels specifically in fixed, extracted specimens. Both antibodies appear to be adsorbed to the ciliary band and to the lining of the gut (arrows). Scale bar is 20 μm in a and b, and 50 μm in c - f.

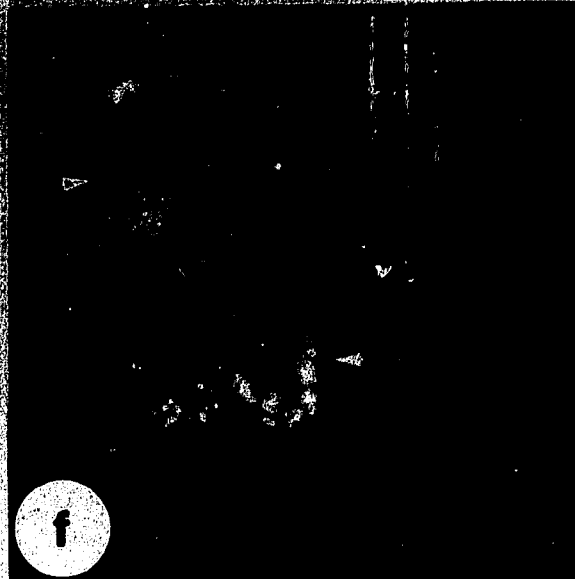
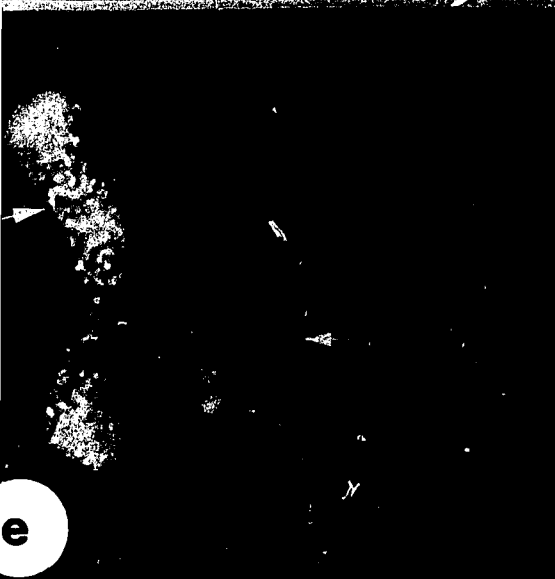


Figure 36. Effect of Sp12 on cells *in vitro*. Cells from dissociated hatched blastulae or early mesenchyme blastulae were plated out in FSW with NRH ascites diluted 1:200 (a, c, e, and g), or with Sp12 ascites diluted 1:200 (b, d, f, and h). NRH incubated cells were labelled with Sp12 (a, c, and e) after fixation to visualize cell processes. Sp12 incubated cells were labelled only with secondary antibody after fixation. **a, b)** Low power view of NRH (a) and Sp12 (b) incubated cells. Many fewer cells adhere in Sp12 treated cultures, and cell shapes are sharply contrasted in the two treatments. **c, d)** Higher magnification view of the cultured cells. NRH incubated cell morphology (c) is indistinguishable from that of cells incubated in FSW alone. Numerous broad pseudopodia extend from each cell and interconnect with those of other cells. In contrast, Sp12 incubated cells (d) are globular and extend a corona of very thin "pencil-lead" filopodia around their perimeter. Cells do not interact, unless they are in tight contact as a doublet. **e, f)** Cells were plated out on cover slips, cultured for 24 hr, then slips were washed and incubated in antibody solutions a further three days. The effect of Sp12 (f) on cell morphology is still apparent even when cells are incubated in antibody after adhesion to the substrate. While not as dramatic as earlier experiments, NRH incubated cells (e) extended large pseudopodia which interconnected cells. Sp12 treated cells (f) remained primarily solitary, and cell processes are generally pencil-lead filopodia. **g, h)** Treated slips were probed with Sp14, a monoclonal antibody against a blastocoelar ECM component in the sea urchin embryo. NRH treated cells secrete Sp14 positive fibres (g). Sp12 treated cells in contrast, show no evidence of a fibrous Sp14-reactive material around them (h). Sp12 treated cells are brightly fluorescent from Sp12 bound in culture, detected with the secondary antibody meant for Sp14 labelling. Scale bars are 20 μm in a and b, and 10 μm in c - h.

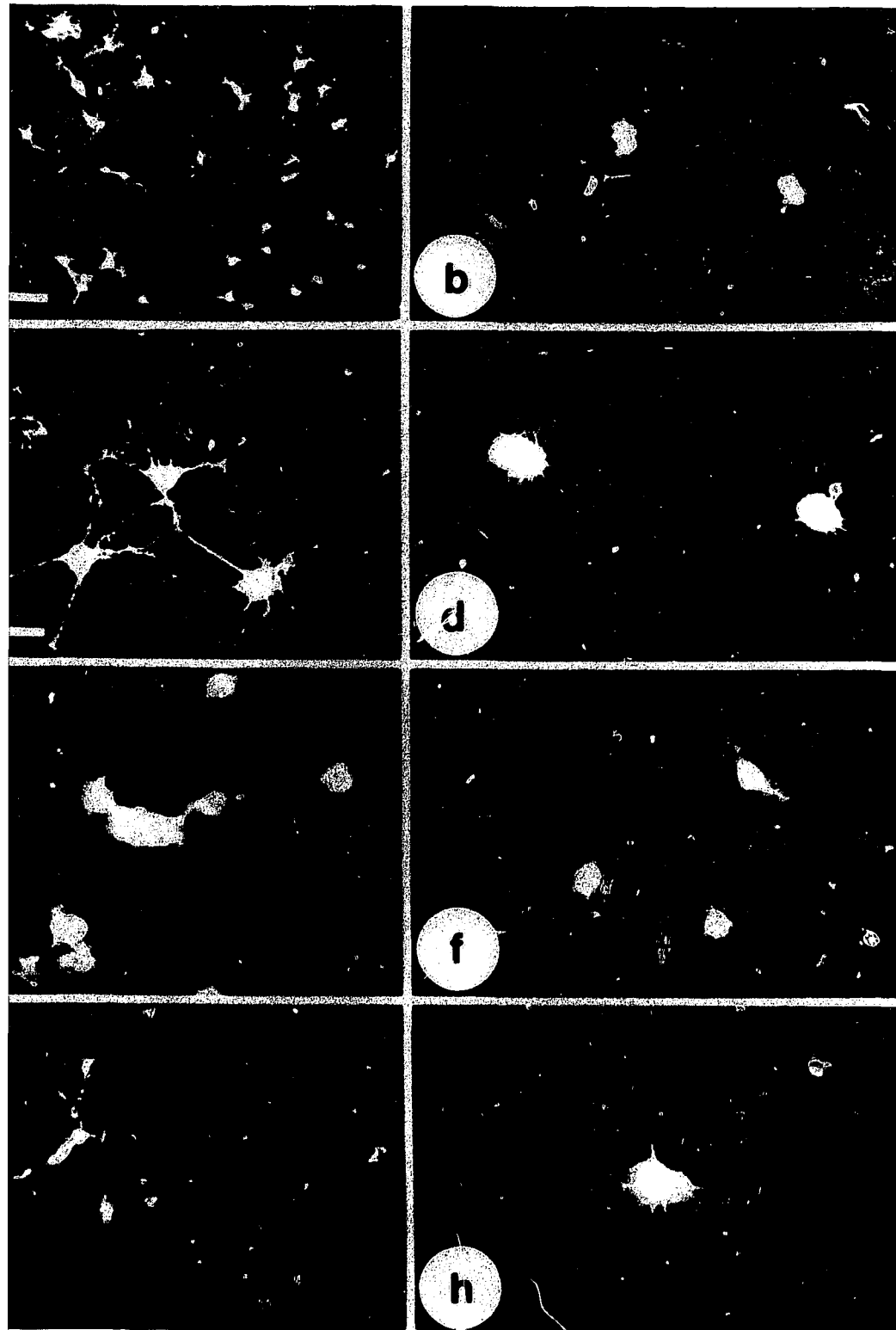


Figure 37. Low concentrations of Sp12 have a subtle effect on cell morphology *in vitro*. Ion exchange purified antibody, diluted sufficiently that it does not label skeletogenic mesenchyme in fixed whole mounts, was used. **a)** NRH incubated cells have typical pseudopodia, and formed extensive mats of interconnected cells. **b)** Sp12 incubated cells are often normal looking, and form extensive cell mats, however, some cells have the characteristic corona of pencil lead filopodia (arrow). Many cells have a vague indication of extending pencil lead filopodia, but they are otherwise normal in morphology (arrowhead). Scale bar is 20 μm .

Figure 38. Increasing concentrations of Sp12 produce increased effects on cell morphology. Protein A purified Sp12 was utilized in this experiment, at concentrations that produced typical immunofluorescence of skeletogenic mesenchyme in fixed whole mounts. **a)** At 15 $\mu\text{g}/\text{ml}$ the filopodia are long and branched, but are as thin as the pencil-lead filopodia. **b)** At 45 $\mu\text{g}/\text{ml}$ specimens have much shorter, typical pencil-lead filopodia, although some cells extended larger processes as well. **c)** 75 $\mu\text{g}/\text{ml}$ produced the same cell morphology as the 1:200 dilution of crude ascites: single cells with a corona of pencil-lead filopodia. **d)** Protein A purified NRH at 65 $\mu\text{g}/\text{ml}$ allowed chains of cells to form via long pseudopodia, with no evidence of filopodia. Scale bar is 10 μm .

Figure 39. Dissociated gastrula stage cells are also perturbed by incubation in Sp12. **a)** Cells from dissociated 48 hr embryos exhibit the same form as 24 hr dissociates when cultured in purified NRH at 60 $\mu\text{g}/\text{ml}$. **b)** Cells incubated in Sp12 at 75 $\mu\text{g}/\text{ml}$ have the round morphology and constrained filopodia of Sp12 incubated cells dissociated at 24 hr. **c)** Sp12 Fab fragments at 17.5 $\mu\text{g}/\text{ml}$ have no effect on cultured cell morphology. These cells extend broad pseudopodia and are extensively interconnected with each other. Scale bar is 10 μm .

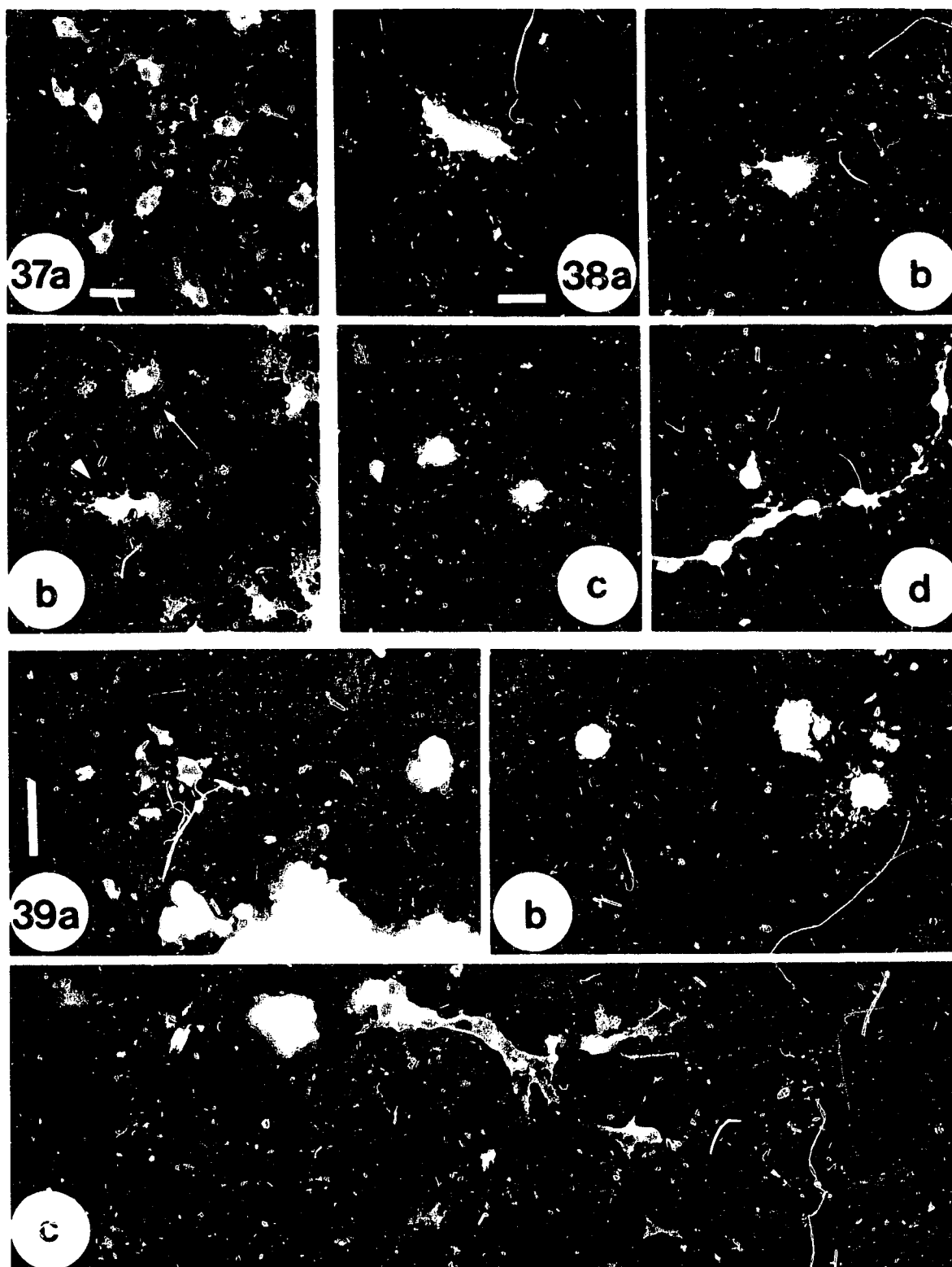
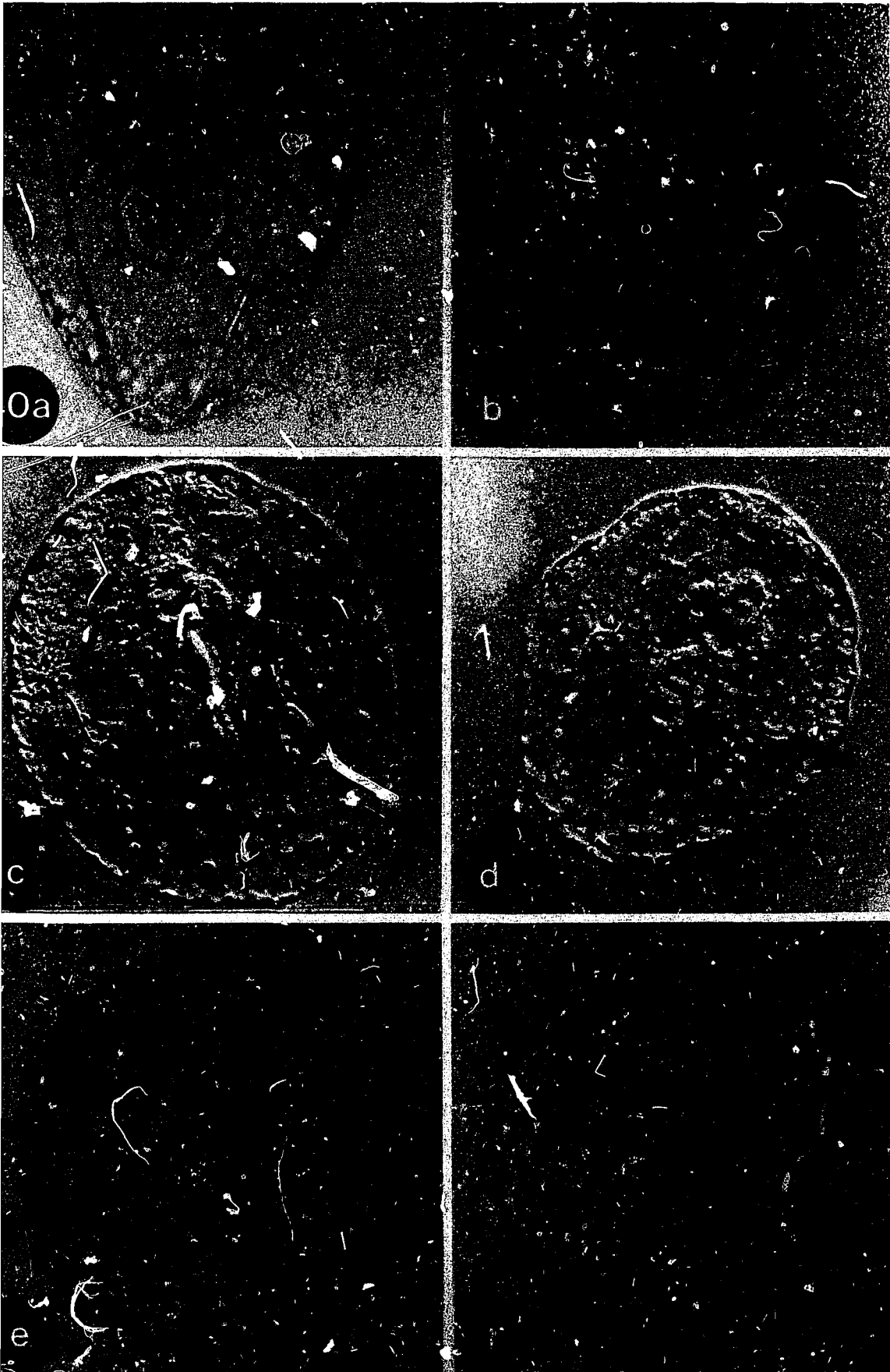


Figure 40. Microinjection of antibody into developing embryos does not produce a specific defect. NRH injected (a, c, and e) embryos are indistinguishable from Sp12 injected embryos (b, d, and f). a, b) NRH injected embryos from this experiment are indistinguishable from uninjected controls. Sp12 injected animals are also morphologically normal (this specimen is mounted in an unusual orientation). c, d) In another experiment disruption of both NRH and Sp12 injected embryos was observed. Larval spicules (sp) are the most commonly malformed tissue in microinjected animals. Other tissues usually appeared normal. Pigment cells (c) migrated into the epidermis and blastocoelar cells (b) took up characteristic positions and morphologies. e, f) Microinjection of crude ascites resulted in tremendous inflation of the blastocoel from oncotic pressure of the injected proteins. Embryos still gastrulated although archenterons (arrows) are displaced to one side of embryos to permit archenteron-stomodeum fusion. Scale bars are 50 μm .



DISCUSSION

Embryos cultured in antibody-FSW

Culturing embryos in dilute Sp12-FSW does not affect development. This result is unexpected given the hyaline layer labelling documented in earlier chapters. Two other anti-hyaline layer monoclonal antibodies dramatically affect morphogenesis in *S. purpuratus*. Adelson and Humphreys (1988) documented morphogenetic arrest of gastrulation and arm formation when embryos are incubated in their hyalin-specific monoclonal antibody. Similar results are reported by Myers (1990) with an antibody against the apical lamina. In that case primary invagination is delayed, resulting in the formation of partial exogastrulae. There are many potential reasons for Sp12's lack of effect. Given the antibody labelling of incubated embryos, the most likely explanation is that Sp12 simply cannot access its epitope in live, intact embryos. Other possibilities to consider include: Sp12 binds an epitope which is removed from the ECM antigen's functional sites; or the Sp12 antigens have no role in morphogenetic events involving the hyaline layer.

It appears from the antibody labelling of incubated embryos that the Sp12 epitope is protected in some manner in the unfixed hyaline layer. Paradoxically, the antibody Sp25, which labels an apical lamina component buried under a thick layer of other hyaline molecules, is able to penetrate to its epitope and bind it without any apparent difficulty (Myers, 1990). The accessibility constraints of Sp12 are consistent with those seen in labelling of fixed specimens. Those preparations require extensive removal of membrane components before clear labelling is apparent. It is interesting, however, that live cell labelling is clear in dissociated cells. Perhaps there is an ECM-membrane molecule apposition which occludes the Sp12 epitope, or sterically prevents antibody binding that is exposed after the ECM is dissolved away. Whatever the cause, it is apparent that if the antibody cannot bind to the antigens it cannot interfere with their function.

While alternate explanations are not required, there are at least two other possible explanations for the lack of an Sp12 effect. Large ECM molecules likely have epitopes which are isolated from functionally essential binding sites, and antibodies which bind to these epitopes would exert little steric effect on such sites. The control antibody in Adelson and Humphreys' (1988) experiments apparently binds such an epitope - it specifically labels hyalin, but has no effect on morphogenesis. It is possible that the Sp12 epitope is removed far enough from active sites on its antigens that steric effects do not interfere with their function.

It is also possible that the Sp12 hyaline layer antigens are not involved in morphogenesis. Farach-Carson *et al.* (1989) have identified the carbohydrate epitope on msp130 that the antibody 1223 recognizes, and postulate that it is involved in calcium sequestration. While this would explain 1223's inhibition of spiculogenesis (Carson *et al.*, 1985), it does not clarify the epitope's role in early development (Farach *et al.*, 1987; Decker *et al.*, 1988). In general carbohydrate moieties are considered essential for gastrulation (Lennarz, 1986), but there is little information on their role in hyaline layer function and early morphogenesis. If Sp12 did have access to its epitope in early embryos, the effect might be too subtle to detect in the crude assays utilized here.

Sp12 effects on cells in vitro

As indicated above, the antibody cannot interfere with antigen it cannot access. *In vitro* culture is a simple way to overcome this obstacle. The *in vitro* technique has been extensively used in assessing the ontogeny and function of the antigen labelled by the monoclonal antibody 1223 (Carson *et al.*, 1985). Spiculogenesis *in vitro* requires horse serum, is a quantifiable characteristic of differentiation, and involves morphogenetic movements (cell migrations) (Decker *et al.*, 1987). A very different set of characters is apparent in cells cultured without horse serum. These are based on the shape of the plated cells (Timourian *et al.*,

1973). Rather than forming syncytial aggregates of rounded, spiculogenic cells lacking extensive pseudopodia, mesenchyme in serum-free cultures form large branching pseudopodia which interconnect cells. While they look like blastocoelar cells, they are probably skeletogenic cells because identical cultures incubated with 2% horse serum form aggregates of rounded cells which grow spicules (data not shown). This contrast in morphology correlates with the conversion of blastocoelar cells into spiculogenic cells in manipulated embryos (Ettensolun and McClay, 1988). It is not known what the horse serum constituents are that stimulate spiculogenic differentiation. However, it is postulated, on the basis of a critical temporal window (36 hr PF - 48 hr PF) and electron microscopy, that serum promotes syncytium formation, a prerequisite to spiculogenesis (McCarthy and Spiegel, 1983c). The intimacy of the cell contacts observed in this study is unknown, but if syncytium formation precedes spiculogenesis, these inert pseudopodial contacts are probably not syncytial.

Other cell morphologies are observed in the serum-free experiments and although many are labelled by Sp12, their relationship to skeletogenic, blastocoelar, and chromogenic mesenchyme is not clear. In cultures derived from plutei the pigment cells often have a slug-like morphology, so this type of cell in earlier cultures may represent pigment cell precursors. Epidermal cells are also slug like after dissociation, although they are not labelled by Sp12. Differences between blastocoelar and skeletogenic cells can not be discerned in cultures from dissociates of any stage.

Sp12 effects on *in vitro* cultures were assessed in a number of different ways. The stage at which embryos are dissociated does not appear to change the Sp12 effect, although cells from earlier stages form more robust cultures. This difference in culture vigor does not influence the results because there is consistency with the control cultures. A similar argument applies to the results from the post-plating

addition of antibody to cultures. The effect of Sp12 on *in vitro* cultures of cells is consistent. Cells are constricted and do not extend pseudopodia. The observation that these cells do not secrete Sp14-reactive material complements the observed morphology. In addition, it has been observed that when Sp12 is added to *in vitro* cultures of migrating pigment cells they immediately (within 5 minutes) round up and stop migrating (Gibson, 1987). Potential explanations of these observations range from a highly specific ligand-receptor interaction to the non-specific crosslinking of immobile cell surface molecules.

The presence of a specific molecular interaction causing this dramatic alteration in cell morphology would be a very interesting finding. It is possible that transmembrane signals from receptors on the cell surface could modulate cortical filament systems which provide the cell with its three dimensional shape. It is now postulated that cell surface and ECM molecules can interact with cytoskeletal molecules to affect cell shape (Horwitz *et al.*, 1986). Such theories can be extended to include inhibition of secretory vesicle shuttling, to explain the lack of Sp14 material around these cells.

At the other extreme, it may be that Sp12's effect on cultured cells is not due to any kind of specific receptor-ligand interaction, but to a nonspecific crosslinking of immobile cell surface molecules. It is clear from the live-cell-labelling experiments that Sp12 antigens are not mobile on the cell surface. They do not cap when incubated with antibody for up to three hours (Chapter 4). If such antigens are crosslinked with antibody while in culture the result could be a cell surrounded by an antibody net. Such a cell might be unable to extend pseudopodia (although fine filopodia may penetrate through the antibody mesh), would not be able to secrete material through vesicle-membrane fusion, and would be immobilized.

Of course, crosslinking of external molecules which are linked to cytoskeletal molecules (as the Sp12 antigens appear to be) could have other intracellular effects.

It may be that the actual impact of Sp12 crosslinking is both nonspecific (the antibody net) and specific (cytoskeletal perturbation). Resolution of this issue was undertaken with univalent Fab fragments of Sp12.

Fab fragment production is relatively easy with Sp12 (the mouse IgG2b immunoglobulin requires little reduction before papain proteolysis), but as outlined in Chapter 2 it resulted in relatively low yields. Low concentration, potential denaturation, and the reduced avidity of a monovalent ligand are all factors to consider in assessing the Fab results. Although these fragments appeared to retain their immunoreactivity, the above three factors probably contributed to the borderline labelling in the Fab *in situ* assay.

Despite these caveats, Fab results must be compared to whole antibody results. Divalent Sp12 at very low concentration exerts a clear effect on cell morphology. The complete absence of any of these constrained characteristics in cells treated with Fab is a clear indication that Sp12's effect on cells requires divalent (hence crosslinking) reagent. While this is evidence for the model of crosslinked, immobile cell surface molecules resulting in constrained cell shape, so receptor mediated events require crosslinking ligands, *e.g.* lymphocyte interactions in the mammalian immune system. Further experiments with Sp12 Fab fragments could confirm these results and resolve the questions that remain.

Injected Sp12 - ambiguous effect

While divalent antibody produced an easily recognized change in cell morphology *in vitro*, this situation does not necessarily reflect the situation within the blastocoel *in vivo*. The blastocoelar environment is much more complex with various ECM materials surrounding cells, epithelial-mesenchymal interactions, and integrated metabolic activities of the various tissues. The lack of difference in experimental and control results in the injection experiments may reflect this increased complexity of the cellular environment. Although the variability in

response can be explained by variations in any of several factors, they appear to have affected both NRH and Sp12 injected animals the same. Sp12 does not exert a consistent, specific, and clear effect of any kind on mesenchyme within the blastocoel.

One of the intriguing effects of antibody injection (both NRH and Sp12) appeared to be disruption of the larval spicules. Spicules do form, but are either malformed or much smaller than normal. Occasionally injected embryos have supernumerary spicules with up to seven triradiate centers around the primary mesenchyme ring. These spicular anomalies are very similar to those described by Iwata and Nakano (1985a) when they injected a monoclonal antibody against a fibronectin-binding acid polysaccharide into sea urchin blastulae. While a specific disruption of spicule morphogenesis would be a very important finding, similar spicule anomalies are observed in NRH injected animals, and in larvae which have not been injected at all. It seems that the skeletogenic mesenchyme pattern is very susceptible to perturbation. This may be a useful morphogenetic system to examine with other techniques or reagents, but establishment of the spicular pattern cannot be specifically investigated with Sp12.

The lack of a specific effect following Sp12 injection is probably attributable to the protective influence of ECM within the blastocoel. As detailed above, the epitope is clearly labelled only in dissociated cells or in permeabilized fixed cells. It is likely that the blastocoel of live embryos does not offer the same accessibility to Sp12 antigens as these situations. While lack of epitope access offers the best explanation, a negative result like this is difficult to translate into a negative conclusion, because there are a number of technical reasons which could also explain it. As it now stands Sp12 *appears* to have no effect on mesenchyme *in vivo*.

CHAPTER 7 CONCLUSIONS

This dissertation documents the characterization of antigens, cells, and tissues labelled by the monoclonal antibody Sp12. Before summarizing these findings it is useful to reiterate the underlying hypotheses this work is based on. Development is a combination of two interwoven processes: differentiation and morphogenesis. While differentiation is very important, unorganized aggregates of differentiated cells do not constitute an organism. The generation of shape which organizes these cells into a coherent whole is morphogenesis. The current paradigm is that cell surface molecules interacting with the ECM, other cell surfaces, and soluble factors direct cells through their morphogenetic movements. The goal of this project was to identify new cell surface molecules in the developing sea urchin, characterize them, and determine their role, if any, in morphogenesis.

Summary of findings - key points in the Sp12 investigation

Sp12 antigens are localized in a novel pattern, spatially and temporally. Initially sequestered in cortical granules, they become a hyaline layer component at fertilization. They are synthesized and secreted by skeletogenic, blastocoelar, and chromogenic mesenchyme, appearing on the cell surface after cells have ingressed and undergone their initial movements. Furthermore, they are present in adults, lining the stereom of the test and spines. Blastocoelar cells are strikingly delineated with Sp12 immunofluorescence, allowing a detailed description of this hitherto ill-defined mesenchyme derivative. They appear to be a connective tissue cell type, analogous to the fibroblast of vertebrates.

A number of different echinoid species share the Sp12 epitope, yet express it on different cell types and at different times. While not pursued here, this offers an

opportunity for investigations of heterochrony and heterotropy - important developmental aspects of evolution.

A unique pattern of temporal changes in the M_r of labelled antigens is documented on western blots of developmental series. Antigens appear, disappear, or change M_r at several points in the ontogeny, although the significance of this remains unknown. The redundant epitope labelled by Sp12 is characterized as an N-linked oligosaccharide, shared by at least ten glycoproteins.

Each one of the diverse array of tissues labelled - hyaline, mesenchyme, and test matrix - appears to incorporate unique as well as shared antigens. Membrane associated antigens and extractable antigens can be separated and analysis indicates that a large M_r subset is easily extractable, while membrane associated antigens form a smaller M_r subset. In a similar way antigens from pigment cells can be separated from blastocoelar and skeletogenic cells which can be separated from cultured skeletogenic cells, revealing discreet subsets of antigens on each cell type.

Membrane associated antigens appear to be anchored and are not mobile within the plasma membrane. The epitope is difficult to obtain access to without stripping away ECM on live cells or dissolving away plasma membrane lipids on fixed specimens.

This last point has made evaluation of the antigen's function difficult because the antibody probably does not have access to its epitope *in vivo*. *In vitro*, the antibody appears to crosslink immobile cell surface antigens and produces a constrained cell form. This effect is not seen when monovalent Fab's are used instead of divalent antibody.

While many of the objectives of this project have been realized, some have remained elusive. As expected, it was not a simple story to unfold, and as is always the case, more questions have been raised than answered. Some of the intriguing questions which remain are addressed below.

Selected points for future investigation

Specific areas to investigate further include: the cellular mechanisms which give the Sp12 antigens their stability at the cell surface; more details about the blastocoelar cell's role in post-gastrula development; using Sp12 to isolate blastocoelar cell specific antigens and antibodies; using Sp12 to examine advanced larvae, adult rudiments, and juveniles; and electron microscopic examination of Sp12 antigens in the adult, looking for clues as to the antigens' function.

The linkage of cell surface molecules to the cytoskeleton offers a mechanism for cellular reaction to environmental stimuli during morphogenesis. Very little is known about this arrangement in sea urchin cells, yet it could be an important link in the understanding of morphogenetic processes. Sp12 antigens appear to be anchored to some cortical structure - elucidation of this connection would be an excellent start to an investigation of cytoskeleton-cell surface molecule-ECM interactions in sea urchin development.

Blastocoelar cells are an intriguing mesenchymal derivative, and the proposed fibroblast analogy deserves further investigation. Establishing cultures enriched in this cell type would be a challenge, yet the opportunities for experimental manipulation of such cultures are extensive. Blastocoelar cell-specific monoclonal antibodies would greatly aid in further characterization of these cells, and Sp12 may be useful in the production of such reagents. Sp12 antigens could be utilized to enrich the immunogen, and blastocoelar cell-specific epitopes which might be recognized could be cloned out.

Very little is known about the larval-adult transition in sea urchins. Sp12's recognition of an epitope and antigens which span metamorphosis provides an opportunity to examine this developmental stage in greater detail. Through labelling of cells and ECM in advanced larvae and their rudiments, as well as in juveniles, definitive connections between larval and adult tissues may be established.

Metamorphosis is an impressive event which utilizes morphogenetic mechanisms on a grand scale, Sp12 labelling may be an effective way to elucidate mesenchyme's role in it. In a related investigation, more detailed examination of the adult tissue labelling of Sp12 could be revealing. Establishing greater resolution of these antigens in test may indicate their function in adults. This information could be extrapolated to provide more clues about the antigens' function in embryos and larvae.

The monoclonal antibody Sp12 provides novel information, enigmatic questions, and beautiful images to those who use it. I hope it is not forgotten too soon.

LITERATURE CITED

- Abed, M. and Crawford, B.J. 1986. Changes in structure and location of extracellular matrix of the blastocoel during development of the starfish *Pisaster ochraceus*. *Canadian Journal of Zoology*, **64**: 1436-1443.
- Adelson, D.L. 1985. *Monoclonal Antibodies to Developmentally Regulated Antigens of the Sea Urchin Embryo*. Ph.D. dissertation, University of Hawaii.
- Adelson, D.L. and Humphreys, T. 1988. Sea urchin morphogenesis and cell-hyalin adhesion are perturbed by a monoclonal antibody specific for hyalin. *Development*, **104**: 391-402.
- Afzelius, B.A. 1956. The ultrastructure of the cortical granules and their products in the sea urchin egg as studied with the electron microscope. *Experimental Cell Research*, **10**: 257-285.
- Akasaka, K. and Terayama, H. 1983. Sulfated glycan present in the EDTA extract of *Hemicentrotus* embryos (mid-gastrula). *Experimental Cell Research*, **146**: 177-185.
- Akasaka, K. and Terayama, H. 1984. A proteoglycan fraction isolated from the EDTA extract of sea urchin (*Hemicentrotus pulcherrimus*) gastrulae stimulates reaggregation of dissociated embryonic cells. *Experimental Cell Research*, **150**: 226-233.
- Akasaka, K., Amemiya, S., and Terayama, H. 1980. Scanning electron microscopical study of the inside of sea urchin embryos (*Pseudocentrotus depressus*). *Experimental Cell Research*, **129**: 1-13.
- Alliegro, M.C., and McClay, D.R. 1988. Storage and mobilization of extracellular matrix proteins during sea urchin development. *Developmental Biology*, **125**: 208-216.
- Alliegro, M.C., Etensohn, C.A., Burdsal, C.A., Erikson, H.P., and McClay, D.R. 1988. Echinonectin: a new embryonic substrate adhesion protein. *Journal of Cell Biology*, **107**: 2319-2327.
- Alliegro, M.C. and Schuel, H. 1988. Immunocytochemical localization of the 35 kDa sea urchin egg trypsin-like protease and its effects upon the egg surface. *Developmental Biology*, **125**: 168-180.
- Amemiya, S. 1989. Development of the basal lamina and its role in migration and pattern formation of primary mesenchyme cells in sea urchin embryos. *Development Growth and Differentiation*, **31**: 131-145.
- Amemiya, S. 1986. Network structure in the blastocoel of developing sea urchin embryos. In: *Progress in Developmental Biology; Part B* (H.C. Slavkin, ed.), pp. 187-190. Alan R. Liss, New York.
- Amemiya, S., Akasaka, K., and Terayama, H. 1982. Scanning electron microscopy of gastrulation in a sea urchin (*Anthocidaris crassispina*). *Journal of Embryology and Experimental Morphology*, **67**: 27-35.

- Angerer, R.C. and Davidson, E.H. 1984. Molecular indices of cell lineage specification in sea urchin embryos. *Science*, **226**: 1153-1160.
- Anstrom, J.A. 1989. Sea urchin primary mesenchyme cells: ingression occurs independent of microtubules. *Developmental Biology*, **131**: 269-275.
- Anstrom, J.A. and Raff, R.A. 1988. Sea urchin primary mesenchyme cells: relation of cell polarity to the epithelial-mesenchymal transformation. *Developmental Biology*, **130**: 57-66.
- Anstrom, J.A., Chin, J.E., Leaf, D.S., Parks, A.L., and Raff, R.A. 1988. Immunocytochemical evidence suggesting heterogeneity in the population of sea urchin cortical granules. *Developmental Biology*, **125**: 1-7.
- Anstrom, J.A., Chin, J.E., Leaf, D.S., Parks, A.L., and Raff, R.A. 1987. Localization and expression of msp130, a primary mesenchyme lineage-specific cell surface protein of the sea urchin embryo. *Development*, **101**: 255-265.
- Barrett, A.J. 1980. The many forms and functions of cellular proteinases. *Federation Proceedings*, **39**: 9-14.
- Bedard, P.A. and Brandhorst, B.P. 1983. Patterns of protein synthesis and metabolism during sea urchin embryogenesis. *Developmental Biology*, **96**: 74-83.
- Benson, S., Benson, N., and Wilt, F. 1986. The organic matrix of the skeletal spicule of sea urchin embryos. *Journal of Cell Biology*, **102**: 1878-1886.
- Benson, S., Jones, E.M.E., Crise-Benson, N., and Wilt, F. 1983. Morphology of the organic matrix of the spicule of the sea urchin larva. *Experimental Cell Research*, **148**: 249-253.
- Benson, S.C. and Sessions, A. 1980. Prolyl hydroxylase activity during sea urchin development. *Experimental Cell Research*, **130**: 467-470.
- Berzofsky, J.A. and Berkower, I.J. Antigen-antibody interactions. In: *Fundamental Immunology* (W.E. Paul, ed.) pp 595-645. Raven Press, N.Y.
- Bisgrove, B.W. and Burke, R.D. 1987. Development of the nervous system of the pluteus larva of *Strongylocentrotus droebachiensis*. *Cell and Tissue Research*, **248**: 335-343.
- Bisgrove, B.W. and Burke, R.D. 1986. Development of serotonergic neurons in embryos of the sea urchin, *Strongylocentrotus purpuratus*. *Development Growth and Differentiation*, **28**: 569-574.
- Bissell, M.J., Hall, H.G., and Parry, G. 1982. How does the extracellular matrix direct gene expression? *Journal of Theoretical Biology*, **99**: 31-68.
- Blankenship, J. and Benson, S. 1984. Collagen metabolism and spicule formation in sea urchin micromeres. *Experimental Cell Research*, **152**: 98-104.

- Boonekamp, P.M. and Pomp, R. 1986. Cation-exchange chromatography - A one-step method to purify monoclonal antibodies from ascites fluid. *Science Tools*, **33**: 5-8.
- Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *Journal of Biological Chemistry*, **256**: 1604-1607.
- Bozhkova, V.P., Khariton, V.Yu., and Chailakhyan, L.M. 1985. Relationship between morphogenesis and intracellular interactions in early sea urchin embryos. *Soviet Journal of Developmental Biology*, **15**: 123-130; translated from *Ontogenez*, **15**: 177-185.
- Burke, R.D. 1985. Actin-mediated retraction of the larval epidermis during metamorphosis of the sand dollar *Dendraster excentricus*. *Cell and Tissue Research*, **239**: 589-597.
- Burke, R.D. 1983. Development of the larval nervous system of the sand dollar, *Dendraster excentricus*. *Cell and Tissue Research*, **229**: 145-154.
- Burke, R.D. 1980a. Morphogenesis of the digestive tract of the pluteus larva of *Strongylocentrotus purpuratus*: shaping and bending. *International Journal of Invertebrate Reproduction*, **2**: 13-21.
- Burke, R.D. 1980b. Development of pedicellariae in the pluteus larva of *Lytechinus pictus* (Echinodermata: Echinoidea). *Canadian Journal of Zoology*, **58**: 1674-1682.
- Burke, R.D. 1978a. *Development of the larval digestive tract of echinoids*. Ph.D. dissertation, Univ. of Alberta.
- Burke, R.D. 1978b. The structure of the nervous system of the pluteus larva of *Strongylocentrotus purpuratus*. *Cell and Tissue Research*, **191**: 233-247.
- Burke, R.D. and Tamboline, C.R. 1990. Ontogeny of an extracellular matrix component of sea urchins and its role in morphogenesis. *Development Growth and Differentiation*, in press.
- Burke, R.D. and Alvarez, C.M. 1988. Development of the esophageal muscles in embryos of the sea urchin *Strongylocentrotus purpuratus*. *Cell and Tissue Research*, **252**: 411-417.
- Burke, R.D. and Gibson, A.W. 1986. Cytological techniques for the study of larval echinoids with notes on methods for inducing metamorphosis. In: *Methods in Cell Biology* (T. Schroeder, ed.), Vol. 27, pp 295-308. Academic Press, New York.
- Burke, R.D. and Chia, F.S. 1980. Morphogenesis of the digestive tract of the pluteus larva of *Strongylocentrotus purpuratus*: sphincter formation. *International Journal of Invertebrate Reproduction*, **2**: 1-12.
- Burnette, W.N. 1981. "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry*, **112**: 195-203.

- Butler, E., Hardin, J., and Benson, S. 1987. The role of lysyl oxidase and collagen crosslinking during sea urchin development. *Experimental Cell Research*, **173**: 174-182.
- Cameron, R.A. and Holland, N.D. 1985. Demonstration of the granular layer and the fate of the hyaline layer during the development of a sea urchin (*Lytechinus variegatus*). *Cell and Tissue Research*, **239**: 455-458.
- Cameron, R.A. and Hinegardner, R.T. 1978. Early events in sea urchin metamorphosis, description and analysis. *Journal of Morphology*, **157**: 21-32.
- Carson, D.D., Farach, M.C., Earles, D.S., Decker, G.L., and Lennarz, W.J. 1985. A monoclonal antibody inhibits calcium accumulation and skeleton formation in cultured embryonic cells of the sea urchin. *Cell*, **41**: 639-648.
- Carson, D.D., Rossignol, D.P., and Lennarz, W.J. 1982. Induction of N-linked glycoprotein synthesis during gastrulation of sea urchin embryos. *Cell Differentiation*, **11**: 323-324.
- Cervello, M. and Matranga, V. 1989. Evidence of a precursor-product relationship between vitellogenin and toposome, a glycoprotein complex mediating cell adhesion. *Cell Differentiation and Development*, **26**: 67-76.
- Chia, F. 1977. Scanning electron microscopic observations of the mesenchyme cells in the larvae of the starfish *Pisaster ochraceus*. *Acta Zoologica (Stockholm)*, **58**: 45-51.
- Chia, F.S. and Burke, R.D. 1978. Echinoderm metamorphosis: fate of larval structures. In: *Settlement and Metamorphosis of Marine Invertebrate Larvae* (F.S. Chia and M.E. Rice, eds.), pp 219-234. Elsevier, N.Y.
- Cloney, R.A., Shaadt, J., and Durden, J.V. 1970. Thermoelectric cooling stage for the compound microscope. *Acta Zoologica*, **51**: 95-98.
- Crawford, B.J. 1988. Regional ultrastructural differences in basal laminae isolated from the starfish *Pisaster ochraceus*. *Development Growth and Differentiation*, **30**: 661-672.
- Crawford, B.J. and Chia, F.S. 1982. Genesis and movement of mesenchyme cells in embryos of the starfish *Pisaster ochraceus*. In: *Echinoderms: Proceedings of the International Conference, Tampa Bay* (J.M. Lawrence, ed.) pp. 505-511. A.A. Balkema, Rotterdam.
- Crise-Benson, N. and Benson, S.C. 1979. Ultrastructure of collagen in sea urchin embryos. *Wilhelm Roux's Archives of Developmental Biology*, **186**: 65-70.
- Czihak, G., ed. 1975. *The sea urchin embryo. Biochemistry and morphogenesis*. Springer-Verlag, N.Y.
- Dan, K. and Okazaki, K. 1956. Cyto-embryological studies of sea urchins. III. Role of the secondary mesenchyme cells in the formation of the primitive gut in sea urchin larvae. *Biological Bulletin*, **110**: 29-42.

- Dan, K. and Ono, T. 1952. Cyto-embryological studies of sea urchins. I. The means of fixation of the mutual positions among blastomeres of sea urchin larvae. *Biological Bulletin*, **102**: 58-72.
- Davidson, E.H. 1989. Lineage-specific gene expression and the regulative capacities of the sea urchin embryo: a proposed mechanism. *Development*, **105**: 421-445.
- Davidson, E.H. 1986. *Gene Activity in Early Development* 3rd ed. Academic Press, New York.
- Davidson, E.H., Hough-Evans, B.R., and Britten, R.J. 1982. Molecular biology of the sea urchin embryo. *Science*, **217**: 17-26.
- Davidson, E.H. and Britten, R.J. 1979. Regulation of gene expression: possible role of repetitive sequences. *Science*, **204**: 1052-1059.
- Decker, G.L. and Lennarz, W.J. 1988. Growth of linear spicules in cultured primary mesenchymal cells of sea urchin embryos is bidirectional. *Developmental Biology*, **126**: 433-436.
- Decker, G.L., Valdizan, M.C., Wessel, G.M., and Lennarz, W.J. 1988. Developmental distribution of a cell surface glycoprotein in the sea urchin *Strongylocentrotus purpuratus*. *Developmental Biology*, **129**: 339-349.
- Decker, G.L., Morrill, J.B., and Lennarz, W.J. 1987. Characterization of sea urchin primary mesenchyme cells and spicules during biomineralization *in vitro*. *Development*, **101**: 297-312.
- DeMay, J.R. 1983. The preparation of immunoglobulin gold conjugates and their use as markers for light and electron microscopic immunocytochemistry. *In: Immunohistochemistry* (A.C. Cuellar, ed.) pp. 347-372. John Wiley and Sons, New York.
- DeSimone, D.W. and Spiegel, M. 1986a. Wheat germ agglutinin binding to the micromeres and primary mesenchyme cells of sea urchin embryos. *Developmental Biology*, **114**: 336-346.
- DeSimone, D.W. and Spiegel, M. 1986b. Concanavalin A and wheat germ agglutinin binding to sea urchin basal laminae. *Roux's Archives of Developmental Biology*, **195**: 433-444.
- DeSimone, D.W., Spiegel, E., and Spiegel, M. 1985. The biochemical identification of fibronectin in the sea urchin embryo. *Biochemistry and Biophysics Research Communications*, **133**: 183-188.
- Drager, B.J., Harkey, M.A., Iwata, M., and Whiteley, A.H. 1989. The expression of embryonic primary mesenchyme genes of the sea urchin, *Strongylocentrotus purpuratus*, in the adult skeletogenic tissues of this and other species of echinoderms. *Developmental Biology*, **133**: 14-23.
- Edelman, G.M. 1986. Cell adhesion molecules in the regulation of animal form and tissue pattern. *Annual Review of Cell Biology*, **2**: 81-116.

- Edelman, G.M., ed. 1985. *Molecular determinants of animal form*. Alan R. Liss, N.Y.
- Edelman, G.M. 1984. Cell adhesion and morphogenesis: the regulator hypothesis. *Proceedings of the National Academy of Science U.S.A.*, **81**: 1460-1464.
- Edelman, G.M. and Gallin, W.J. 1987. Cell adhesion as a basis of pattern in embryonic development. *American Zoologist*, **27**: 645-656
- Ekblom, P., Vestweber, D., and Kemler, R. 1986. Cell-matrix interactions and cell adhesion during development. *Annual Review of Cell Biology*, **2**: 27-47.
- Ettensohn, C.A. 1985. Gastrulation in the sea urchin embryo is accompanied by the rearrangement of invaginating epithelial cells. *Developmental Biology*, **112**: 383-396.
- Ettensohn, C.A. 1984. Primary invagination of the vegetal plate during sea urchin gastrulation. *American Zoologist*, **24**: 571-588.
- Ettensohn, C.A. and McClay, D.R. 1988. Cell lineage conversion in the sea urchin embryo. *Developmental Biology*, **125**: 396-409.
- Ettensohn, C.A. and McClay, D.R. 1987. A new method for isolating primary mesenchyme cells of the sea urchin embryo: panning on wheat germ agglutinin-coated dishes. *Experimental Cell Research*, **168**: 431-438.
- 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. *Developmental Biology*, **117**: 330-391.
- Farach, M.C., Vaidizan, M., Park, H.R., Decker, G.L., and Lennarz, W.J. 1987. Developmental expression of a cell surface protein involved in calcium uptake and skeleton formation in sea urchin embryos. *Developmental Biology*, **122**: 320-331.
- Farach-Carson, M.C., Carson, D.D., Collier, J.L., Lennarz, W.J., Park, H.R., and Wright, G.C. 1989. A calcium binding, asparagine-linked oligosaccharide is involved in skeleton formation in the sea urchin embryo. *Journal of Cell Biology*, **109**: 1289-1299.
- Fink, R.D. and McClay, D.R. 1985. Three cell recognition changes accompany the ingress of sea urchin primary mesenchyme cells. *Developmental Biology*, **107**: 66-74.
- Freeman, G. 1989. The factors that promote the development of symmetry properties in aggregates from dissociated echinoid embryos. *Roux's Archives of Developmental Biology*, **197**: 394-405.
- Fuchs, E. and Green, H. 1980. Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell*, **19**: 1033-1042.
- Fujiki, Y., Hubbard, A.L., Fowler, S., and Lazarow, P.B. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: Application to endoplasmic reticulum. *Journal of Cell Biology*, **93**: 97-102.

- Gache, C., Niman, H.L., and Vacquier, V.D. 1983. Monoclonal antibodies to the sea urchin egg vitelline layer inhibit fertilization by blocking sperm adhesion. *Experimental Cell Research*, **147**: 75-84.
- Galau, G.A., Klein, W.H., Davis, M.M., Wold, B.J., Britten, R.J., and Davidson, E.H. 1976. Structural gene sets active in embryos and adult tissues of the sea urchin. *Cell*, **7**: 487-505.
- Galfre, G. and Milstein C. 1981. Preparation of monoclonal antibodies: Strategies and procedures. In: *Methods in Enzymology* (J.J. Langone and H. van Vukanis, eds.), Vol.73, pp. 3-47. Academic Press, New York.
- Galileo, D. 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. *Journal of Morphology*, **185**: 387-402.
- Gefter, M.L., Margulies, D.H., and Scharff, M.D. 1977. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. *Somatic Cell Genetics*, **3**: 231-236.
- Gibbins, J.R., Tilney, L.G., and Porter, K.R. 1969. Microtubules in the formation and development of the primary mesenchyme in *Arbacia punctulata*. *Journal of Cell Biology*, **41**: 201-226.
- Gibson, A.W. 1987. *Morphogenesis and differentiation of pigment cells in embryos of the sea urchin Strongylocentrotus purpuratus*. Ph.D. dissertation, Univ. of Victoria.
- Gibson, A.W. and Burke, R.D. 1985. The origin of pigment cells in embryos of the sea urchin *Strongylocentrotus purpuratus*. *Developmental Biology*, **107**: 414-419.
- Gibson, A.W. and Burke, R.D. 1987. Migratory and invasive behavior of pigment cells in normal and animalized sea urchin embryos. *Experimental Cell Research*, **173**: 546-557.
- Gibson, A.W. and Burke, R.D. 1988. Localization and characterization of an integral membrane protein antigen expressed by pigment cells in embryos of the sea urchin *Strongylocentrotus purpuratus*. *Development Growth and Differentiation*, **30**: 283-292.
- Giudice, G. 1973. *Developmental biology of the sea urchin embryo*. Academic Press, N.Y.
- Giudice, G. 1962. Restitution of whole larvae from disaggregated cells of sea urchin embryos. *Developmental Biology*, **2**: 402-411.
- Giudice, G and Mutolo, V. 1970. Reaggregation of dissociated cells of sea urchin embryos. *Advances in Morphogenesis*, **8**: 115-158.
- Goding, J.W. 1986. *Monoclonal Antibodies: Principles and Practice*, 2nd ed. Academic Press, N.Y.

- Gustafson, T.U. 1975. Cellular behaviour and cytochemistry in early stages of development. *In: The Sea Urchin Embryo: Biochemistry and Morphogenesis* (G. Czihak, ed.), pp. 233-266. Springer-Verlag, New York.
- Gustafson, T.U. 1969. Cell recognition and cell contacts during sea urchin development. *In: Cellular Recognition* (R.T. Smith and R.A. Good, eds.) pp. 47-60. Appleton-Century-Crofts, New York.
- Gustafson, T. 1963. Cellular mechanisms in the morphogenesis of the sea urchin embryo - Cell contacts within the ectoderm and between mesenchyme and ectoderm cells. *Experimental Cell Research*, **32**: 570-589.
- Gustafson, T.U. and Toneby, M.I. 1971. How genes control morphogenesis: the role of serotonin and acetylcholine in morphogenesis. *American Scientist*, **59**: 452-462.
- Gustafson, T.U. and Wolpert, L. 1967. Cellular movement and contact in sea urchin morphogenesis. *Biological Reviews*, **42**: 447-498.
- Gustafson, T. and Wolpert, L. 1963. Studies on the cellular basis of morphogenesis in the sea urchin embryo: formation of the coelom, the mouth, and the primary pore canal. *Experimental Cell Research*, **29**: 561-582.
- Gustafson, T. and Wolpert, L. 1961. Cellular mechanisms in the morphogenesis of the sea urchin larva: the formation of arms. *Experimental Cell Research*, **22**: 509-520.
- Hall, H.G. and Vacquier, V.D. 1982. The apical lamina of the sea urchin embryo: Major glycoproteins associated with the hyaline layer. *Developmental Biology*, **89**: 168-178.
- Hardin, J. 1968. The role of secondary mesenchyme cells during gastrulation studied by laser ablation. *Development*, **103**: 317-324.
- Hardin, J.D. and Cheng, L.Y. 1986. The mechanisms and mechanics of archenteron elongation during sea urchin gastrulation. *Developmental Biology*, **115**: 490-501.
- Harkey, M.A. 1983. Determination and differentiation of micromeres in the sea urchin embryo. *In: Time, Space, and Pattern in Embryonic Development* (W.R. Jeffery and R.A. Raff, eds.) pp. 131-155. Alan R. Liss, New York.
- Harkey, M.A., Whiteley, H.R., and Whiteley, A.H. 1988. Coordinate accumulation of five transcripts in the primary mesenchyme during skeletogenesis in the sea urchin embryo. *Developmental Biology*, **125**: 381-395.
- Harkey, M.A. and Whiteley, A.H. 1982. Cell-specific regulation of protein synthesis in the sea urchin gastrula: a two-dimensional electrophoretic study. *Developmental Biology*, **93**: 453-462.
- Harkey, M.A. and Whiteley, A.H. 1980. Isolation, culture, and differentiation of echinoid primary mesenchyme cells. *Wilhelm Roux's Archives of Developmental Biology*, **189**: 111-122.

- Harris, A.K., Wild, P., and Stopak, D. 1980. Silicone rubber substrata: a new wrinkle in the study of cell locomotion. *Science*, **208**: 177-179.
- Hay, E.D., ed. 1981. *Cell biology of the extracellular matrix*. Plenum, N.Y.
- Heatfield, B.M. 1971. Growth of the calcareous skeleton during regeneration of spines of the sea urchin, *Strongylocentrotus purpuratus* (Stimpson): a light and scanning electron microscopic study. *Journal of Morphology*, **134**: 57-90.
- Heatfield, B.M. and Travis, D.F. 1975a. Ultrastructural studies of regenerating spines of the sea urchin *Strongylocentrotus purpuratus*. I. Cell types without spherules. *Journal of Morphology*, **145**: 13-50.
- Heatfield, B.M. and Travis, D.F. 1975b. Ultrastructural studies of regenerating spines of the sea urchin *Strongylocentrotus purpuratus*. II. Cell types with spherules. *Journal of Morphology*, **145**: 51-72.
- Heifetz, A. and Lennarz, W.J. 1979. Biosynthesis of N-glycosidically linked glycoproteins during gastrulation of sea urchin embryos. *Journal of Biological Chemistry*, **254**: 6119-6127.
- Herbst, C. 1900. Über das Auseinandergelien von Furchungs und Gewebzellen in Kalk-freien Medium. *Wilhelm Roux' Archiv für Entwicklungsmechanik der Organismen*, **9**: 424-463.
- Herbst, C. 1904. Über die zur Entwicklung der Seeigellarven notwendigen anorganischen Stoffe, Ihre Rolle and ihre Verthbarkeit. II Teil Die rolle der notwendigen anorganischen Stoff. *Wilhelm Roux' Archiv für Entwicklungsmechanik der Organismen*, **17**: 305-520.
- Honjo, T. 1981. Rearrangements of immunoglobulin genes during differentiation and evolution. *Immunological Review*, **59**: 33-67.
- Horstadius, S. 1975. Isolation and transplation experiments. *In: The Sea Urchin Embryo: Biochemistry and Morphogenesis* (G. Czihak, ed.), pp. 364-406. Springer-Verlag, New York.
- Horstadius, S. 1973. *Experimental Embryology of Echinoderms*. Clarendon Press, Oxford.
- Horstadius, S. 1939. The mechanics of sea urchin development studied by operative methods. *Biological Review*, **14**: 132-179.
- Horwitz, A., Duggan, K., Puck, C., Beckerle, M.C., and Burridge, K. 1986. Interaction of plasma membrane fibronectin receptor with talin - a transmembrane linkage. *Nature*, **320**: 531-533.
- Hylander, B.L. and Summers, R.G. 1982. An ultrastructural immunocytochemical localization of hyalin in the sea urchin egg. *Developmental Biology*, **93**: 368-380.
- Hyman, L.H. 1955. *The Invertebrates: Echinodermata, the Coelomate Bilateria (Vol. IV)*. McGraw-Hill, New York.

- Immers, J. and Runnstrom, J. 1965. Further studies on the effects of deprivation of sulphate on the early development of the sea urchin *Paracentrotus lividus*. *Journal of Embryology and Experimental Morphology*, **14**: 289-305.
- Ishimoda-Takagi, T., Chino, I., and Sato, H. 1984. Evidence for the involvement of muscle tropomyosin in the contractile elements of the coelom-esophagus complex in sea urchin embryos. *Developmental Biology*, **105**: 365-376.
- Iwata, M. and Nakano, E. 1984. Cell-to-substratum adhesion of dissociated embryonic cells of the sea urchin, *Pseudocentrotus depressus*. *Roux's Archives of Developmental Biology* **193**: 71-77.
- Iwata, M. and Nakano, E. 1985a. Fibronectin-binding acid polysaccharide in the sea urchin embryo. *Roux's Archives of Developmental Biology*, **194**: 377-384.
- Iwata, M. and Nakano, E. 1985b. Enhancement of spicule formation and calcium uptake by monoclonal antibodies to fibronectin-binding acid polysaccharide in cultured sea urchin embryonic cells. *Cell Differentiation*, **17**: 57-62.
- Kane, R.E. 1973. Hyaline release during normal sea urchin development and its replacement after removal at fertilization. *Experimental Cell Research*, **81**: 301-311.
- Karp, G.C. and Solursh, M. 1974. Acid mucopolysaccharide metabolism, the cell surface, and primary mesenchyme cell activity in the sea urchin embryo. *Developmental Biology*, **41**: 110-123.
- Karp, G.C. and Solursh, M. 1985a. Dynamic activity of the filopodia of sea urchin embryonic cells and their role in directed migration of the primary mesenchyme *in vitro*. *Developmental Biology*, **112**: 276-283.
- Karp, G.C. and Solursh, M. 1985b. *In vitro* fusion and separation of sea urchin primary mesenchyme cells. *Experimental Cell Research*, **158**: 554-557.
- Katow, H. 1987. Inhibition of cell surface binding of fibronectin and fibronectin-promoted cell migration by synthetic peptides in sea urchin primary mesenchyme cells *in vitro*. *Development Growth and Differentiation*, **29**: 579-589.
- Katow, H. 1986. Behavior of sea urchin primary mesenchyme cells in artificial extracellular matrices. *Experimental Cell Research*, **162**: 401-410.
- Katow, H. and Amemiya, S. 1986. Behaviour of primary mesenchyme cells *in situ* associated with ultrastructural alteration of the blastocoelic material in the sea urchin, *Anthocidaris crassispina*. *Development Growth and Differentiation*, **28**: 31-42.
- Katow, H. and Hayashi, M. 1985. Role of fibronectin in primary mesenchyme migration. *Journal of Cell Biology*, **101**: 1487-1491.
- Katow, H., Yamada, K.M., and Solursh, M. 1982. Occurrence of fibronectin on the primary mesenchyme cell surface during migration in the sea urchin embryo. *Differentiation*, **22**: 120-124.

- Katow, H. and Solursh, M. 1982. *In situ* distribution of concanavalin A-binding sites in mesenchyme blastulae and early gastrulae of the sea urchin *Lytechinus pictus*. *Experimental Cell Research*, **139**: 171-180.
- Katow, H. and Solursh, M. 1981. Ultrastructural and time-lapse studies of primary mesenchyme cell behaviour in normal and sulfate-deprived sea urchin embryos. *Experimental Cell Research*, **136**: 233-245.
- Katow, H. and Solursh, M. 1979. Ultrastructure of blastocoel material in blastulae and gastrulae of the sea urchin *Lytechinus pictus*. *Journal of Experimental Zoology*, **210**: 561-567.
- Kawabe, T.T., Armstrong, P.B., and Pollock, E.G. 1981. An extracellular fibrillar matrix in gastrulating sea urchin embryos. *Developmental Biology*, **85**: 509-515.
- Keller, R. and Hardin, J. 1987. Cell behaviour during active cell rearrangement: evidence and speculations. *Journal of Cell Science Supplement*, **8**: 369-393.
- Kennett, R. 1979. Cell fusion. *Methods in Enzymology*, **58**: 345-359.
- Kew, D.A. 1984. *Cultured sea urchin embryo epithelial and mesenchymal cells differ in adhesiveness, lectin agglutinability and lectin receptor mobility*. Ph.D. dissertation, Wesleyan University.
- Kerkis, A.Yu. and Isaeva, V.V. 1984. Electron microscopic study of spiculogenesis in a culture of embryonic cells of the sea urchin *Strongylocentrotus nudus*. *Soviet Journal of Developmental Biology*, **15**: 24-30. Translated from *Ontogenez*, **15**: 34-40.
- Kinoshita, T. and Okazaki, K. 1984. *In vitro* study on morphogenesis of sea urchin larval spicule: adhesiveness of cells. *Zoological Science*, **1**: 433-443.
- Kinoshita, S. and Saiga, H. 1979. The role of proteoglycan in the development of sea urchins. 1. Abnormal development of sea urchin embryos caused by the disturbance of proteoglycan synthesis. *Experimental Cell Research*, **123**: 229-236.
- Klein, W.H., Hardin, J.H., and Brandhorst, B.F. 1987. Specific proteins: calcium binding proteins in the embryonic ectodermal cells of sea urchins. *Methods in Enzymology*, **139**: 354-363.
- Koch, G.L.E. 1981. The anchorage of cell surface receptors to the cytoskeleton. In: *International Cell Biology 1980-1981* (H.G. Schweiger, ed.) pp. 321-330. Springer-Verlag, N.Y.
- Kohler, G. and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **256**: 495-497.
- Kominami, T. 1985. The role of cell adhesion in the differentiation of mesendodermal tissues in the starfish, *Asterina pectinifera*. *Development Growth and Differentiation*, **27**: 679-688.

- Kominami, T. 1984. Allocation of mesendodermal cells during early embryogenesis in the starfish, *Asterina pectinifera*. *Journal of Embryology and Experimental Morphology*, **84**: 177-190.
- Kondo, K. and Sakai, H. 1971. Demonstration and preliminary characterization of reaggregation-promoting substances from embryonic sea urchin cells. *Development Growth and Differentiation*, **13**: 1-14.
- Kornfeld, R. and Kornfeld, S. 1980. Structure of glycoproteins and their oligosaccharide units. In: *The Biochemistry of Glycoproteins and Proteoglycans* (W.J. Lennarz, ed.) pp. 1-34. Plenum Press, New York.
- Krach, S.W., Green, A., Nicolson, G.L., and Oppenheimer, S.B. 1974. Cell surface changes during sea urchin embryonic development monitored by quantitative agglutination with plant lectins. *Experimental Cell Research*, **84**: 191-198.
- Krstic, R.V. 1984. *Illustrated Encyclopedia of Human Histology*. Springer-Verlag, N.Y.
- Kyoizumi, S. and Kominami, T. 1980. Inhibitory effect of concanavalin A on the cell-cell adhesion during development of the starfish *Asterina pectinifera*. *Experimental Cell Research*, **128**: 323-331.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.
- Lallier, R. 1972. Effects of concanavalin A on the development of sea urchin egg. *Experimental Cell Research*, **72**: 157-163.
- Lane, M.C. and Solursh, M. 1988. Dependence of sea urchin primary mesenchyme cell migration on xyloside- and sulphate-sensitive cell surface-associated components. *Developmental Biology*, **127**: 78-87.
- Langelan, R.E. and Whiteley, A.H. 1985. Unequal cleavage and the differentiation of echinoid primary mesenchyme. *Developmental Biology*, **109**: 464-475.
- Lau, J.T.Y. and Lennarz, W.J. 1983. Regulation of sea urchin glycoprotein mRNAs during embryonic development. *Proceedings of the National Academy of Science U.S.A.*, **80**: 1028-1032.
- Leaf, D.S., Anstrom, J.A., Chin, J.E., Harkey, M.A., Showman, R.M., and Raff, R.A. 1987. Antibodies to a fusion protein identify a cDNA clone encoding msp130, a primary mesenchyme-specific cell surface protein of the sea urchin embryo. *Developmental Biology*, **121**: 29-40.
- Lee, G.F., Fanning, E.W., Small, M.P., and Hille, M.B. 1989. Developmentally regulated proteolytic processing of a yolk glycoprotein complex in embryos of the sea urchin, *Strongylocentrotus purpuratus*. *Cell Differentiation and Development*, **26**: 5-18.
- Lennarz, W.J. 1986. Regulation of glycoprotein synthesis during development of the sea urchin embryo. In: *Developmental Biology: A Comprehensive Synthesis, Vol. III: The Cell Surface in Development and Cancer* (M.S. Steinberg, ed.), pp. 59-79. Plenum Press, New York.

- Lennarz, W.J. 1983. Glycoprotein synthesis and embryonic development. *Critical Reviews in Biochemistry*, **14**: 257-272.
- Lovtrup-Rein, H. and Lovtrup, S. 1984. Sulfated polysaccharides and cell differentiation in the sea urchin embryo. *Experimental Cell Biology*, **52**: 383-388.
- Lundgren, B. 1973. Surface coatings of the sea urchin larva as revealed by ruthenium red. *Journal of Submicroscopic Cytology*, **5**: 61-70.
- Markel, K. and Roser, U. 1983. The spine tissues in the echinoid *Eucidaris tribuloides*. *Zoomorphology*, **103**: 25-41.
- Matranga, V., Kuwasaki, B. and Noll, H. 1986. Functional characterization of toposomes from sea urchin blastula embryos by a morphogenetic cell aggregation assay. *European Molecular Biology Organization Journal*, **5**: 3125-3132.
- Matsumoto, E., Tonegawa, Y., and Ishihara, K. 1984. Studies on the localization and activities of concanavalin A reactive glycoproteins on the cell surface of sea urchin embryos. *Journal of Experimental Zoology*, **232**: 157-165.
- MacBride, E.W. 1914. The development of *Echinocardium cordatum*: the external features of the development. *Quarterly Journal of Microscopic Science*, **59**: 471-486.
- MacBride, E.W. 1903. The development of *Echinus esculentus*, together with some points in the development of *E. miliaris* and *E. acutus*. *Philosophical Transactions of the Royal Society, London, series B*, **195**: 285-325.
- McCarthy, R.A. and Burger, M.M. 1987. *In vivo* embryonic expression of laminin and its involvement in cell shape change in the sea urchin *Sphaerechinus granularis*. *Development*, **101**: 659-671.
- McCarthy, R.A., Beck, K., and Burger, M.M. 1987. Laminin is structurally conserved in the sea urchin basal lamina. *European Molecular Biology Organization Journal*, **6**: 1587-1593.
- McCarthy, R.A. and Spiegel, M. 1983a. Protein composition of the nyaline layer of sea urchin embryos and reaggregating cells. *Cell Differentiation*, **13**: 93-102.
- McCarthy, R.A. and Spiegel, M. 1983b. The enhancement of reaggregation of sea urchin blastula cells by exogenous proteins. *Cell Differentiation*, **13**: 103-114.
- McCarthy, R.A. and Spiegel, M. 1983c. Serum effects on the *in vitro* differentiation of sea urchin micromeres. *Experimental Cell Research*, **149**: 433-441.
- McClay, D.R. 1979. Surface antigens involved in interactions of embryonic sea urchin cells. In: *Current Topics in Developmental Biology*, Vol. 13, pp. 199-214. Academic Press, New York.

- McClay, D.R., Coffman, J.A., and Wessel, G.M. 1987. Cell recognition during sea urchin gastrulation. Expression of molecules associated with morphogenesis. *In: Molecular Approaches to Developmental Biology* (E.H. Davidson, ed.), pp. 231-241. Alan R. Liss, New York.
- McClay, D.R. and Etensohn, C.A. 1987a. Cell adhesion in morphogenesis. *Annual Review of Cell Biology*, 3: 319-345.
- McClay, D.R. and Etensohn, C.A. 1987b. Cell recognition during sea urchin gastrulation. *In: Genetic Regulation of Development*, (W.F. Loomis, ed.) pp 111-128. Alan R. Liss, N.Y.
- McClay, D.R. and Matrangola, V. 1986. On the role of calcium in the adhesion of embryonic sea urchin cells. *Experimental Cell Research*, 165: 152-164.
- McClay, D.R. and Fink, R.D. 1982. Sea urchin hyalin: Appearance and function in development. *Developmental Biology*, 92: 285-293.
- McClay, D.R. and Marchase, R.B. 1979. Separation of ectoderm and endoderm from sea urchin pluteus larvae and demonstration of germ layer-specific antigens. *Developmental Biology*, 71: 289-296.
- McClay, D.R. and Chambers, A.F. 1978. Identification of four classes of cell surface antigens appearing at gastrulation in sea urchin embryos. *Developmental Biology*, 63: 179-186.
- McClay, D.R., Chambers, A.F., and Warren, R.H. 1977. Specificity of cell-cell interactions in sea urchin embryos. Appearance of new cell-surface determinants at gastrulation. *Developmental Biology*, 56: 343-355.
- McKearn, T.J. 1980. Cloning of hybridoma cells by limiting dilution in fluid phase. *In: Monoclonal antibodies. Hybridomas: a new dimension in biological analyses* (R.H. Kennett, T.J. McKearn, and K.B. Bechtol, eds.) p 374. Plenum, N.Y.
- Milstein, C. and Lennox, E. 1980. The use of monoclonal antibody techniques in the study of developing cell surfaces. *Current Topics in Developmental Biology*, 14: 1-32.
- Mintz, G.R., DeFrancesco, S., and Lennarz, W.J. Spicule formation by cultured embryonic cells from the sea urchin. *Journal of Biological Chemistry*, 256: 13105-13111.
- Mizoguchi, H., Fujiwara, A., and Yasumasu, I. 1989. Synthesis of collagen-like proteins in embryonic organs of the sea urchin, *Hemicentrotus pulcherrimus*. *Development Growth and Differentiation*, 31: 189-196.
- Morrill, J.B. 1986. Scanning electron microscopy of embryos. *In: Methods in Cell Biology* (T. Schroeder, ed.), Vol. 27, pp 264-293. Academic Press, New York.
- Morrill, J.B. and Doty, S.D. 1986. Mechanical and cellular aspects of gastrulation in the sea urchin *Lytechinus variegatus*. *In: Progress in Developmental Biology* (H.C. Slavkin, ed.) pp 97-100. Alan R. Liss, N.Y.

- Myers, R.L. 1990. *The apical lamina and its role in the development of Strongylocentrotus purpuratus*. Master's thesis, University of Victoria.
- Nelson, S.H. and McClay, D.R. 1988. Cell polarity in sea urchin embryos: reorientation of cells occurs quickly in aggregates. *Developmental Biology*, **127**: 235-247.
- Nislow, C. and Morrill, J.B. 1988. Regionalized cell division during sea urchin gastrulation contributes to archenteron formation and is correlated with the establishment of larval symmetry. *Development Growth and Differentiation*, **30**: 483-499.
- Nocente-McGrath, C., Brenner, C.A., and Ernst, S.G. 1989. Endo16, a lineage-specific protein of the sea urchin embryo, is first expressed just prior to gastrulation. *Developmental Biology*, **136**: 264-272.
- Noll, H., Mataranga, V., Cascino, D., and Vittorelli, L. 1979. Reconstitution of membranes and embryonic development in dissociated blastula cells of the sea urchin by reinsertion of aggregation-promoting membrane proteins extracted with butanol. *Proceedings of the National Academy of Science U.S.A.*, **76**: 288-292.
- Noll, H., Mataranga, V., Cervello, M., Humphreys, T., Kuwasaki, B., and Adelson, D. 1985. Characterization of toposomes from sea urchin blastula cells: a cell organelle mediating cell adhesion and expressing positional information. *Proceedings of the National Academy of Science U.S.A.*, **82**: 8062-8066.
- Okazaki, K. 1975a. Normal development to metamorphosis. In: *The Sea Urchin Embryo: Biochemistry and Morphogenesis* (G. Czihak, ed.), pp.177-232. Springer-Verlag, New York.
- Okazaki, K. 1975b. Spicule formation by isolated micromeres of the sea urchin embryo. *American Zoologist*, **15**: 567-581.
- Okazaki, K. 1960. Skeleton formation of sea urchin larvae. II. Organic matrix of the spicule. *Embryologia*, **5**: 283-320.
- Oppenheimer, S.B. and Meyer, J.T. 1982. Carbohydrate specificity of sea urchin blastula adhesion component. *Experimental Cell Research*, **139**: 451-455.
- Parks, A.L., Parr, B.A., Chin, J.E., Leaf, D.S., and Raff, R.A. 1988. Molecular analysis of heterochronic changes in the evolution of direct developing sea urchins. *Journal of Evolutionary Biology*, **1**: 27-44.
- Pearson, T.W., Galfre, G., Ziegler, A., and Milstein, C. 1977. A myeloma hybrid producing antibody specific for an allotypic determinant on "IgD-like" molecules of the mouse. *European Journal of Immunology*, **7**: 684.
- Pehrson, J.R., and Cohen, L.H. 1986. The fate of the small micromeres in sea urchin development. *Developmental Biology*, **113**: 522-526.
- Pittman, D. and Ernst, S.G. 1984. Developmental time, cell lineage, and environment regulate the newly synthesized proteins in sea urchin embryos. *Developmental Biology*, **106**: 236-242.

- Pointer, D. 1978. *Blastocoelic fluid of the sea urchin embryo: its components and their significance in development*. Ph.D. dissertation. U.C. Berkeley.
- Rapraeger, A.C. and Epel, D. 1981. The appearance of an extracellular arylsulfatase during morphogenesis of the sea urchin *Strongylocentrotus purpuratus*. *Developmental Biology*, **88**: 269-278.
- Richardson, W., Kitajima, T., Wilt, F., and Benson, S. 1989. Expression of an embryonic spicule matrix gene in calcified tissues of adult sea urchins. *Developmental Biology*, **132**: 266-269.
- Roe, J.L., Park, H.R., Strittmatter, W.J., and Lennarz, W.J. 1989. Inhibitors of metalloendoproteases block spiculogenesis in sea urchin primary mesenchyme cells. *Experimental Cell Research*, **181**: 542-550.
- Runnstrom, J., Horstadius, S., Immers, J., and Fudge-Mastrangelo, M. 1964. An analysis of the role of sulfate in the embryonic differentiation of the sea urchin (*Paracentrotus lividus*). *Revue Suisse Zoologie*, **71**: 21-54.
- Ryberg, E. 1977. The nervous system of the early echinopluteus. *Cell and Tissue Research*, **179**: 157-167.
- Ryberg, E. and Lundgren, B. 1977. Extra-ectodermal strands in the ciliated bands of the echinopluteus. *Development Growth and Differentiation*, **19**: 299-308.
- Schneider, E.G. 1985. Cell-cell recognition and adhesion during embryogenesis in the sea urchin: demonstration of species-specific adhesion among *Arbacia punctulata*, *Lytechinus variegatus*, and *Strongylocentrotus purpuratus*. *Biological Bulletin*, **168**: 61-74.
- Schneider, E.G., Nguyen, H.T., and Lennarz, W.J. 1978. The effect of tunicamycin, an inhibitor of protein glycosylation, on embryonic development in the sea urchin. *Journal of Biological Chemistry*, **253**: 2348-2355.
- Schroeder, T.E. 1988. Contact-independent polarization of the cell surface and cortex of free sea urchin blastomeres. *Developmental Biology*, **125**: 255-264.
- Schroeder, T.E., ed. 1986a. *Echinoderm Gametes and Embryos*. Methods in Cell Biology, Vol. 27. Academic Press, N.Y.
- Schroeder, T.E. 1986b. The egg cortex in early development of sea urchins and starfish. In: *Developmental Biology: A Comprehensive Synthesis, Vol. II: The Cellular Basis of Morphogenesis* (L.W. Browder, ed.), pp. 59-100. Plenum Press, New York.
- Schroeder, T.E. 1981. Development of a "primitive" sea urchin (*Eucidaris tribuloides*): irregularities in the hyaline layer, micromeres, and primary mesenchyme. *Biological Bulletin*, **161**: 141-151.
- Shimizu, K., Noro, N., and Matsuda, R. 1988. Micromere differentiation in the sea urchin embryo: Expression of primary mesenchyme cell specific antigen during development. *Development Growth and Differentiation*, **30**: 35-47.

- Showman, R.M. and Foerder, C.A. 1979. Removal of the fertilization membrane of sea urchin embryos employing aminotriazole. *Experimental Cell Research*, **120**: 253-255.
- Smith, R.H. 1984. *Sea urchin primary mesenchyme development: inactivation of cell-cell adhesion molecules and appearance of cell-substrate adhesion molecules*. Ph.D. dissertation. Wesleyan University.
- Solursh, M. 1986. Migration of sea urchin primary mesenchyme cells. In: *Developmental Biology: A Comprehensive Synthesis, Vol. II: The Cellular Basis of Morphogenesis* (L.W. Browder, ed.), pp. 391-431. Plenum Press, New York.
- Solursh, M. and Lane, M.C. 1988. Extracellular matrix triggers a directed cell migratory response in sea urchin primary mesenchyme cells. *Developmental Biology*, **130**: 397-401.
- Solursh, M., Mitchell, S.L. and Katow, H. 1986. Inhibition of cell migration in sea urchin embryos by β -D-xyloside. *Developmental Biology*, **118**: 325-332.
- Solursh, M. and Katow, H. 1982. Initial characterization of sulphated macromolecules in the blastocoels of mesenchyme blastulae of *Strongylocentrotus purpuratus* and *Lytechinus pictus*. *Developmental Biology*, **94**: 326-336.
- Spiegel, E., Howard, L. and Spiegel, M. 1989. Extracellular matrix of sea urchin and other marine invertebrate embryos. *Journal of Morphology*, **199**: 71-92.
- Spiegel, E. and Spiegel, M. 1986. Cell-cell interactions during sea urchin morphogenesis. In: *Developmental Biology: A Comprehensive Synthesis, Vol. II: The Cellular Basis of Morphogenesis* (L.W. Browder, ed.), pp. 195-240. Plenum Press, New York.
- Spiegel, E. and Howard, L. 1983. Development of cell junctions in sea urchin embryos. *Journal of Cell Science*, **62**: 27-48.
- Spiegel, E., Burger, M., and Spiegel, M. 1983. Fibronectin and laminin in the extracellular matrix and basement membrane of sea urchin embryos. *Experimental Cell Research*, **144**: 47-55.
- Spiegel, E., Burger, M., and Spiegel, M. 1980. Fibronectin in the developing sea urchin embryo. *Journal of Cell Biology*, **87**: 309-313.
- Spiegel, E. and Spiegel, M. 1979. The hyaline layer is a collagen containing extracellular matrix in sea urchin embryos and reaggregating cells. *Experimental Cell Research*, **123**: 434-441.
- Spiegel, E. and Spiegel, M. 1975. The reaggregation of dissociated embryonic sea urchin cells. *American Zoologist*, **15**: 583-606.
- Spiegel, M. and Burger, M.M. 1982. Cell adhesion during gastrulation: a new approach. *Experimental Cell Research*, **139**: 377-382.

- Spiro, R.G. 1966. Characterization of carbohydrate units of glycoproteins. *Methods in Enzymology*, **8**: 26-52.
- Stearns, L.W. 1974. *Sea urchin development. Cellular and molecular aspects.* Dowden, Hutchinson and Ross, Inc. Stroudsburg, Penn.
- Steck, T.L. 1974. The organization of proteins in the human red blood cell membrane. *Journal of Cell Biology*, **62**: 1-19.
- Steck, T.L. and Yu, J. 1973. Selective solubilization of proteins from red blood cell membranes by protein perturbants. *Journal of Supramolecular Structure*, **1**: 220-232.
- Stephens, L., Hardin, J., Keller, R., and Wilt, F. 1986. The effects of aphidicolin on morphogenesis and differentiation in the sea urchin embryo. *Developmental Biology*, **118**: 64-69.
- Stopak, D. and Harris, A.K. 1982. Connective tissue morphogenesis by fibroblast traction. *Developmental Biology*, **90**: 383-398.
- Strathman, R.R. 1989. Existence and functions of a gel-filled primary body cavity in development of echinoderms and hemichordates. *Biological Bulletin*, **176**: 25-31.
- Strathman, M. 1968. *Methods in Development Series I. General Procedures for Echinodermata - Echinoidea.* Friday Harbour Laboratories, Friday Harbour.
- Swift, D.M., Sikes, S., and Wheeler, A.P. 1986. Analysis and function of organic matrix from sea urchin tests. *Journal of Experimental Zoology*, **240**: 65-73.
- Takeichi, M. 1988. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development*, **102**: 639-655.
- Thom, R. 1983. *Mathematical models of morphogenesis.* Translated by W.M. Brookes and D. Rand. Ellis Horwood, Chichester.
- Timourian, H., Clothier, G., and Watchmaker, G. 1973. Reaggregation of sea urchin blastula cells. I. Intrinsic differences in the blastula cells. *Developmental Biology*, **31**: 252-263.
- Tonegawa, Y. 1984. A Ca^{++} binding protein responsible for Ca^{++} dependant cell association of sea urchin embryos. *Development Growth and Differentiation*, **27**: 181-182.
- Tonegawa, Y. 1973. Isolation and characterization of a particulate cell aggregation factor from sea urchin embryos. *Development Growth and Differentiation*, **14**: 337-352.
- Tonegawa, Y., Doi, T., and Suzuki, T. 1986. A Ca^{2+} binding protein and an endogenous lectin from sea urchin embryos. In: *Progress in Developmental Biology; Part B* (H.C. Slavkin, ed.), pp. 211-214. Alan R. Liss, New York.

- Trimmer, J.S., Ebina, Y., Schackmann, R.W., Meinhof, C.G., and Vaquier, V.D. 1987. Characterization of a monoclonal antibody that induces the acrosome reaction of sea urchin sperm. *Journal of Cell Biology*, **105**: 1121-1128.
- Trinkaas, J.P. 1984. *Cells into organs: the forces that shape the embryo*. 2nd ed. Prentice-Hall, N.J.
- Truschel, M.R., Chambers, S.A., and McClay, D.R. 1986. Two antigenically distinct forms of B-1,3-glucanase in sea urchin development. *Developmental Biology*, **117**: 277-285.
- Urban, S., Nislow, C., and Spiegel, M. 1988. The origin of skeleton-forming cells in the sea urchin embryo. *Roux's Archives of Developmental Biology*, **197**: 447-456.
- Vermaasubramanian, K. and Solursh, M. 1984. Adhesive and migratory behaviour of normal and sulfate-deficient sea urchin cells *in vitro*. *Experimental Cell Research*, **154**: 421-431.
- Watanabe, M., Bertolini, D.R., Kew, D., and Turner, R.S. 1982a. Changes in the nature of cell adhesions of the sea urchin embryo. *Developmental Biology*, **91**: 278-285.
- Watanabe, M., Bertolini, D.R., Schnitman, J.R., and Turner, R.S. 1982b. Reconstitution of embryo-like structures from sea urchin embryo cells: distinction between cell-cell and cell-substrate associations. *Differentiation*, **21**: 79-85.
- Watson, J.D., Hopkins, N.H., Roberts, J.W., Steitz, J.A., and Weiner, A.M. 1987. *Molecular biology of the gene*. 4th ed. Benjamin/Cummings, Menlo Park, Calif.
- Weiner, S. 1985. Organic matrixlike macromolecules associated with the mineral phase of sea urchin skeletal plates and teeth. *Journal of Experimental Zoology*, **234**: 7-15.
- Welply, J.K., Lau, J.T., and Lennarz, W.J. 1985. Developmental regulation of glycosyltransferases involved in synthesis of N-linked glycoproteins in sea urchin embryos. *Developmental Biology*, **107**: 252-258.
- Wessel, G.M. and McClay, D.R. 1987. Gastrulation in the sea urchin embryo requires deposition of crosslinked collagen within the extracellular-matrix. *Developmental Biology*, **121**: 149-165.
- Wessel, G.M. and McClay, D.R. 1985. Sequential expression of germ-layer specific molecules in the sea urchin embryo. *Developmental Biology*, **111**: 451-463.
- Wessel, G.M., Marchase, R.B., and McClay, D.R. 1984. Ontogeny of the basal lamina in the sea urchin embryo. *Developmental Biology*, **103**: 235-245.
- Wessells, N.K. 1977. *Tissue interactions and development*. Benjamin-Cummings, Menlo Park, California.

- Westin, M. 1972. The occurrence of stage specific antigens during early sea urchin development. *Journal of Experimental Zoology*, **179**: 207-214.
- Wilt, F.H. 1987. Determination and morphogenesis in the sea urchin embryo. *Development*, **100**: 559-575.
- Wilt, F.H., Benson, S., Benson, N. 1987. Spicule formation in sea urchin embryos. *In: Molecular Approaches to Developmental Biology* (E.H. Davidson, ed.), pp. 223-230. Alan R. Liss, New York.
- Wolpert, L. 1981. Positional information, pattern formation, and morphogenesis. *In: Morphogenesis and Pattern Formation* (T.G. Connelly, L.L. Brinkley, and B.M. Carlson, eds) pp 5-20. Raven Press, N.Y.
- Wolpert, L. and Stein, W.D. 1984. Positional information and pattern formation. *In: Pattern Formation: A Primer in Developmental Biology* (G.M. Malacinski and S.V. Bryant, eds) pp 3-17. Macmillan, N.Y.
- Woodward, M.P., Young, W.W., and Bloodgood, R.A. 1985. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *Journal of Immunological Methods*, **78**: 143-153.
- Wray, G.A. and McClay, D.R. 1988. The origin of spicule forming cells in a 'primitive' sea urchin (*Eucidaris tribuloides*) which appears to lack primary mesenchyme cells. *Development*, **103**: 305-315.
- Wray, G.A. and McClay, D.R. 1989. Molecular heterochronies and heterotropies in early echinoid development. *Evolution*, **43**: 803-813.
- Yamada, K.M. 1983. Cell surface interactions with extracellular materials. *Annual Review of Biochemistry*, **52**: 761-799.
- Yazaki, I. 1984. The egg originated and local distribution of the surface of sea urchin embryo cells detected by immunofluorescence. *Acta Embryologica Morphologica Experimentia*, **5**: 3-22.
- Yu, J., Fischman, D.A., and Steck, T.L. 1973. Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. *Journal of Supramolecular Structure*, **1**: 233-245.