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Analysis of compaction, allocation, and outgrowth in the early mouse embryo

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Analysis of Compaction, Allocation, and Outgrowth in the Early Mouse Embryo

by

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DISSERTATION

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Dedication

This work is dedicated to my mother, Jean Sutherland, and to my father, Thomas Sutherland: their strength, determination, and courage I will always strive to emulate.

Abstract

The experiments described in this thesis examine the processes of compaction, allocation, and outgrowth in the early mouse embryo. The polarization hypothesis of early mouse development proposes that the divergence of the trophectoderm and inner cell mass of the blastocyst originates from the structural polarization of the 8-cell blastomeres during compaction, followed by segregation of the different regions of the 8-cell blastomere into different populations of 16-cell blastomeres by delaminatory division (Johnson et al., 1981). Examination of the process of compaction by cinemicrography, and by the use of cytoskeleton-disrupting agents showed it to be a variable, progressive event, dependent on microfilaments both for cell flattening and for polarization of surface microvilli. Examination of the subsequent cleavage division by cinemicrography revealed that there are three major planes of division, and made possible the determination of their relative frequencies during fourth cleavage. The observed frequencies are consistent with the polarization hypothesis, however, the lack of correlation between division order and division plane orientation during fourth cleavage indicates that mechanical forces within the embryo may also play a role in determining blastomere fate. Attachment and trophoblast outgrowth on substrates of extracellular matrix molecules was found to involve a group of 140 kD glycoproteins similar to those of the integrin family of extracellular matrix receptors characterized in avian cells. Addition to the culture medium of a synthetic peptide containing the Arg-Gly-Asp tripeptide cell recognition sequence of fibronectin inhibited trophoblast outgrowth, but not attachment, on both laminin and fibronectin. Immunoprecipitation of ^{125}I surface-labelled embryos revealed that the 140 kD glycoproteins are exposed on the surfaces of embryos during outgrowth formation, but are not detectable immediately after hatching. Immunofluorescence experiments showed that the 140 kD glycoproteins and the cytoskeletal proteins vinculin and talin are enriched on the cell

processes and ventral surfaces of trophoblast cells in embryo outgrowths, in patterns similar to those seen in fibroblasts, and consistent with their role in adhesion of the trophoblast cells to the substratum. The results described in this thesis provide new information on the formation and early function of trophectoderm in mouse development.

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List of Abbreviations

AB blastomere	First 2-cell blastomere to divide during second cleavage
Arg	Arginine
Asp	Aspartic acid
BHK	Baby hamster kidney
BSA	Bovine serum albumin
CB	Cytochalasin B
CD blastomere	Second 2-cell blastomere to divide during second cleavage
CM	Colcemid
Col IV	Collagen type IV
DME	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ECMr	Extracellular matrix receptor
EDTA	Ethylenediaminetetraacetic acid
Fn	Fibronectin
FNR	Fibronectin receptor
Gly	Glycine
GRGDSP	Glycine-Arginine-Glycine-Aspartic acid-Serine-Proline
GRGESP	Glycine-Arginine-Glycine-Glutamic acid-Serine-Proline
hCG	Human chorionic gonadotropin
HRP	Horseradish peroxidase
ICM	Inner cell mass
ICR	Institute of Cancer Research
JAR cells	Human trophoblast cells
kD	Kilodaltons
LC	Lumicolchicine
LM	Light microscopy
Ln	Laminin
µCi	Microcuries
µl	Microliters

µm	Micrometers
mm	Millimeters
MMTE cells	Mouse mammary tumor epithelial cells
M_r	Relative mobility
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulfonylfluoride
SEM	Scanning electron microscopy
S.E.M.	Standard error of the mean
TE	Trophectoderm
TM	Tunicamycin
TNB	Tris-NP40 buffer
U	Units of activity
Vnc	Vinculin
WGA	Wheat germ agglutinin

Introduction

The pre- and periimplantation stages of mouse development comprise a number of very simple but important developmental events that are responsible for establishing the basic structure of the embryo. During the early cleavage stages, two populations of blastomeres, differing by virtue of their position in the morula and early blastocyst, become established. The fourth cleavage division produces these two populations of blastomeres- the outer cells, which face the external medium, and the inner cells which lack contact with the external medium and are enclosed by the outer cells. The the inner and outer blastomeres are distinct from one another not only by their position, but also by their morphology and behavior (Reeve and Ziomek, 1981; Ziomek and Johnson, 1981; Ziomek et al., 1982; Fleming and Pickering, 1985; Maro et al., 1985). Morphologically, outer cells of the morula are larger than inner cells , exhibit polarized organization of internal cellular components and surface microvilli, and are only adhesive on a restricted area of their surface. In contrast, the inner cells of the morula are generally smaller, exhibit uniform organization of cellular organelles and microvilli, and are very adhesive on all parts of their surface (Handyside, 1981; Reeve and Ziomek, 1981; Johnson and Ziomek, 1981; Ziomek and Johnson, 1981; Kimber et al., 1982; Fleming and Pickering, 1985; Maro et al., 1985). At the blastocyst stage, these differences become more fully developed. Outer cells of the morula become the flattened epithelial trophectoderm (TE) of the blastocyst, while the inner cells of the morula become the eccentrically placed inner cell mass (ICM). TE cells are flattened, epithelial cells with functional tight junctions and the ability to pump ions, and thus fluid, vectorially (McLaren and Smith, 1978; Wiley and Eglitis, 1981; reviewed in Kaye, 1986). ICM cells are smaller and rounded, without specialized junctional connections between themselves or with the TE (Calarco and Brown, 1969; Ducibella et al., 1975; Magnuson et al., 1977). By the blastocyst

stage, qualitative differences are seen in protein synthetic patterns between TE and ICM (Van Blerkom et al., 1976; Handyside and Johnson, 1978). The divergence of the two cell populations of the expanded blastocyst continues through the rest of development in the implanted embryo. TE cells give rise to placental structures, while ICM cells give rise to the embryo proper and its associated membranes (Gardner et al., 1973; Papaioannou, 1982; Rossant et al., 1983; reviewed in Rossant, 1986).

Thus the segregation of inner and outer cells that first occurs in the morula represents the first and most basic tissue differentiation of the development of the mouse embryo, namely the separation of the progenitor cells of the embryo from those of the placenta. In this thesis, two basic issues concerning the differentiation of two cell types from a common progenitor have been examined. The first is how the inner and outer cells of the morula become segregated from one another, and the second is how the differences between these two cell populations are generated and maintained.

To examine the process of segregation of the inner and outer cells of the 16-cell stage embryo, or morula, one needs to look one stage earlier, at the 8-cell stage. From the time of fertilization until the 8-cell stage, development of the mouse embryo is fairly simple and straightforward. The blastomeres cleave slowly and asynchronously, without growth (Kelly et al., 1978). At the 8-cell stage, the formerly spherical blastomeres flatten on one another, maximizing the area of intercellular contact, and creating a rounded embryo (Lewis and Wright, 1935; Borghese and Cassini, 1963; Mulnard, 1967; Ducibella and Anderson, 1975). This process of cell flattening is referred to as compaction. Reorganization of both internal cellular components and cell surface microvilli into a polarized configuration occurs concurrently with the flattening of the 8-cell blastomeres (Handyside, 1980; Reeve, 1981; Reeve and Ziomek, 1981; Fleming et al., 1986; Fleming and Pickering, 1985;

Johnson and Maro, 1985; reviewed in Johnson and Maro, 1986). Intercellular junctions also begin to form at this time between the formerly loosely-adherent blastomeres (Calarco and Brown, 1969; Ducibella et al., 1975; Magnuson et al., 1977; Lo and Gilula, 1979; McLachlin et al., 1983). Following compaction, the embryo will undergo two more cleavage divisions before undergoing its second major morphogenetic event, which is formation of the blastocoel.

The polarization phase of compaction generates marked asymmetries in the blastomeres of the 8-cell stage embryo. Actin and tubulin become localized to the cytocortical region (Ducibella et al., 1977; Lehtonen and Badley, 1980; Johnson and Maro, 1984; Houliston et al., 1987), clathrin and endosomes become localized subapically (Fleming and Pickering, 1985; Maro et al., 1985), the nucleus reportedly moves more basally (Reeve and Kelly, 1983), and the surface distributions of microvilli and various lectin receptors become restricted to the apex of each blastomere (Handyside, 1980; Reeve, 1981; Reeve and Ziomek, 1981). Some asymmetries are also apparent as early as the 2- and 4-cell stages, notably in the distribution of myosin (Sobel, 1983a, b, 1984), spectrin (Sobel and Alliegro, 1985) and various cell-surface moieties (Mulnard and Huygens, 1978; Izquierdo et al., 1980; Handyside et al., 1987).

The polar asymmetry of the 8-cell blastomeres appears to be maintained through fourth cleavage and reflected in the differences between the inner and outer cells of the morula. As described above, the inner cells are smaller, more adhesive, have few microvilli and lectin-binding sites, and have a random organization of cellular components; outer cells are larger, less adhesive and have a polarized distribution of both cellular components and surface microvilli and lectin binding sites. How is the asymmetry of the 8-cell stage blastomere translated into the differences between the

inner and outer cells of the morula, and by extension, into the differences between TE and ICM?

The simplest explanation is that the 8-cell blastomeres undergo delaminatory division during fourth cleavage, and thereby segregate their structurally different regions into different populations of blastomeres. Theoretically, such a delamination during fourth cleavage could be entirely responsible for the foundation of the inner cell population (Johnson, 1981; Johnson and Ziomek, 1981). However, experiments done on intact embryos demonstrate that this cannot be the case (Gearhart et al., 1982; Balakier and Pedersen, 1982; Fleming, 1987; Dyce et al., 1987). Firstly, not all of the 8-cell blastomeres produce outer-inner pairs of daughter blastomeres at the 16-cell stage. Injection of 8-cell blastomeres with horseradish peroxidase (HRP) and examination of the position of daughter 16-cell blastomeres reveals that delaminatory division occurs only about half the time (Gearhart et al., 1982; Balakier and Pedersen, 1982; Pedersen et al., 1986). Similar results are seen when 8-cell blastomeres labelled with fluorescent beads are recombined with unlabelled blastomeres and the positions of daughter cells examined (Fleming, 1987; Garbutt et al., 1987). In addition, observation of the position of cytoplasmic bridges between sister 16-cell blastomeres shows that two positional variants exist, corresponding to cleavage planes that are either perpendicular to or tangential to the surface of the blastomere (Soltynska, 1982). Secondly, 16-cell blastomeres also contribute descendants to the inner cell population (Pedersen et al., 1986; Fleming, 1987; Dyce et al., 1987). It is only after the fifth cleavage division that the outer cells no longer produce inner descendants (Cruz and Pedersen, 1985; Pedersen, 1986; Dyce et al., 1987).

An issue that has never been addressed is whether the orientation of the division planes of 8-cell blastomeres determines the positional fate of their daughter

cells in the 16-cell morula. Indeed, the division planes of 8-cell blastomeres have never been examined in the intact embryo. Chapter One of this thesis describes the cinemicrographic observation of fourth cleavage, with an analysis of the orientation of the planes of division and their frequencies. In addition, since several studies have reported a correlation between division order and extent of allocation of descendants to the inner cell population (Kelly et al., 1978; Surani and Barton, 1982; Garbutt et al., 1987), the possible connection between division order and orientation of the division plane was also examined.

If the basis for the segregation of the inner and outer cells of the morula is the production of non-equivalent daughter cells by delaminatory division, then one must consider how the asymmetries of the 8-cell blastomeres are generated, and whether they are important. Since polarization of the 8-cell blastomeres occurs during compaction, it becomes important to examine the cellular mechanisms that control compaction. This has been approached in several ways. First, what controls the overt processes of cell flattening and intracellular reorganization? In other cell types the cytoskeleton is responsible for effecting changes in cell shape and polarity (Viamontes et al., 1979; Vasiliev et al., 1970; reviewed in Trinkaus, 1984), and in most respects this is the case for the mouse embryo as well. Cell flattening and polarization are rapidly reversed by treatment with cytochalasin, implicating microfilaments in both processes (Pratt et al., 1981, 1982; Fleming et al., 1986). In contrast, inhibitory agents for the other major cytoskeletal system, microtubules, do not affect either cell flattening or polarization (Ducibella et al., 1975; Ducibella, 1982; Pratt et al., 1982; Johnson and Maro, 1985) except to accelerate their rate (Johnson and Maro, 1985). Treatment of embryos with Taxol, an agent which stabilizes polymerized tubulin and causes uncontrolled proliferation of microtubules, does block compaction (Johnson and Maro, 1985). These results suggest that microtubules do not actively participate in

either cell flattening or polarization, but that their organization must be modified in order for compaction to occur.

Cell surface adhesion must also be a component in the compaction process, since it is during compaction that adhesions between blastomeres become prominent and intercellular junctions are first formed (Calarco and Brown, 1969; Ducibella and Anderson, 1975; Magnusen et al., 1978; McLachlin et al., 1983; Goodall and Johnson, 1984). Treatment of embryos with tunicamycin, a drug that blocks N-linked glycosylation of proteins, has inhibitory effects on compaction (Maylie-Pfenninger, 1979; Surani, 1979; Atienza-Samols et al., 1980; Pratt et al., 1982). Incubation of embryos in medium containing antibodies to cell-surface glycoproteins will inhibit or rapidly reverse compaction, (Kemler et al., 1977; Hyafil et al., 1981; Damsky et al., 1983; Shirayoshi et al., 1983; Peyreiras et al., 1983; Johnson et al., 1986) and will slow the rate of polarization (Shirayoshi et al., 1983; Johnson et al., 1986). The involvement of specific types of lectin-carbohydrate interactions is indicated by studies showing that 8-cell blastomeres will spread on beads coated with certain lectins (Kimber et al., 1981; Kimber and Surani, 1982). More dramatically, incubation of 2-cell embryos in medium containing the lectin wheat germ agglutinin (WGA) will cause premature cell flattening and cavitation (Johnson, 1985). It is not known whether WGA treatment also induces premature polarization.

The effects that inhibitors of microfilaments, microtubules, and protein glycosylation have on the ultrastructure of the embryo and on the process of compaction have never been carefully examined. In Chapter Two of this thesis, I present the results of an scanning electron microscope analysis of the effects of these inhibitors on compaction and polarization. These results were published previously

(Sutherland and Calarco-Gillam, 1983) and have been reinterpreted here in light of subsequent findings (Maro and Pickering, 1984; Johnson and Maro, 1985).

Clearly, then, the cytoskeleton and cell surface components are involved in the implementation of compaction and polarization. What controls the onset of these two processes is less clear. By applying inhibitors of DNA and protein synthesis to embryos at various times prior to the 8-cell stage, it has been determined that all the necessary factors for effecting cell flattening are present by the late 2-cell or early 4-cell stage (Kidder and McLaughlin, 1985; Levy et al., 1986). In contrast, the factors required for gap-junctional assembly (McLaughlin et al., 1983; Kidder and McLaughlin, 1985) and polarization (Levy et al., 1986) are not present until the late 4-cell stage. Treatment with inhibitors of protein synthesis at the early 4-cell stage actually induced premature compaction and surface polarization (Levy et al., 1986) suggesting the presence of a restraining factor (Levy et al., 1986; Johnson, 1985; Johnson and Maro, 1986).

It is clear that the differences between outer and inner cells do not denote differentiation or even determination of either cell population. Isolation of 16-cell blastomeres of either type followed by their reaggregation with various combinations of other blastomeres demonstrates that both inner and outer cells have the ability to generate either TE or ICM (Ziomek and Johnson, 1982; Johnson and Ziomek, 1983). Entire 16-cell embryos can even be reaggregated such that their descendants lie exclusively in the ICM (Hillman et al., 1972). Isolated groups of outer cells will generate ICM (Rossant and Vijh, 1980; Ziomek et al., 1982) and isolated groups of inner cells will regenerate TE (Handyside, 1978; Hogan and Tilly, 1978; Spindle, 1978). In spite of all this, some constraints have been found, primarily for the outer, polar cells (Ziomek and Johnson, 1982). When an outer cell is reaggregated such that

it is completely enclosed, it will still give rise to at least one descendant that is an outer, polarized cell (Ziomek and Johnson, 1982; Johnson and Ziomek, 1982; Randle, 1982; Surani and Handyside, 1983), whereas inner cells subjected to the same procedures show no such consistent production of outer, polarized descendants (Ziomek and Johnson, 1982; Johnson and Ziomek, 1982; Randle, 1982; Surani and Handyside, 1983). In addition, labelled 8- and 16-cell outer blastomeres always have at least one descendant that is an outer cell (Pedersen et al., 1986; Garbutt et al., 1987). It thus appears that even at the early stages of segregation of these two cell types, the blastomeres containing the apical region of the 8-cell blastomeres are strongly biased toward the production of outer descendants, and thus are biased towards the trophectoderm lineage.

Two hypotheses have been put forward to explain the differentiation of TE and ICM. The first, set forward by Tarkowski and Wroblewska (1967) holds that the TE and ICM of the mouse blastocyst become determined and differentiate according to their position in the 16-cell morula. Blastomeres on the outside create a special environment for the blastomeres on the inside, causing the latter to differentiate into ICM. The predictions of this "inside-outside hypothesis" are that blastomeres are equivalent and totipotent until the 16-cell stage, when some become enclosed, and that changing the position of a given blastomere will alter its fate (Tarkowski and Wroblewska, 1967; reviewed in Pedersen, 1986). The second hypothesis was first articulated by Johnson et al. (1981). Known as the "polarization hypothesis", it proposes that the initial step in the differentiation of TE and ICM is the polarization of 8-cell blastomeres, which results in the creation of non-equivalent regions in each. Delaminatory division during fourth cleavage would then segregate these non-equivalent regions, generating two populations of blastomeres that differ from one another in terms of their cytoplasmic inheritance from their 8-cell progenitor.

These two hypotheses make different predictions about the state of determination of the 16-cell blastomeres. The inside-outside hypothesis predicts that all 16-cell blastomeres are initially equivalent, and differentiate according to their different environments (inside or outside) at the 16-cell stage. The polarization hypothesis, on the other hand, proposes that the inner and outer 16-cell blastomeres are different from the moment of their formation, and their position and ultimate fate are guided by their phenotype (polar or apolar) at the 16-cell stage. In fact, what may be happening is that the phenotypic characteristics of the inner and outer 16-cell blastomeres predispose them to assume certain positions in the morula and early blastocyst. The different environments encountered by the blastomeres in each of these positions then act to further direct the differentiation of the blastomeres, resulting in the fully determined TE and ICM of the late blastocyst. The question of whether the phenotypic differences between the inner and outer cells of the morula arise from differential inheritance due to delaminatory division during fourth cleavage is addressed in Chapter Two of this thesis.

Trophectoderm Function during Implantation

Adhesive interactions, between cells, and between cells and their surrounding extracellular matrix, are important in development. The notion that tissue affinities, perhaps based in large measure on adhesive properties of cells to one another and to extracellular matrix, organize embryonic tissue movements is a fundamental concept emerging in large part from the work of Holtfreter (1939; Townes and Holtfreter, 1955). More recently, specific changes in cell-cell and cell-matrix interactions have been documented in the ingression of both sea urchin primary mesenchyme cells (Fink and McClay, 1985) and chick endocardial cells (Markwald et al., 1975,1977). Cell-matrix interactions are involved in the morphogenesis of branching organs, such as

salivary gland (Banerjee et al., 1977; Bernfield and Banerjee, 1982) and lung (Bluemink et al., 1976; Grant et al., 1983). In addition, cellular migration through an extracellular matrix is a fundamental part of the morphogenesis of many other tissues including the cornea (Toole and Trelstad, 1971; Bard et al., 1975), the endocardial cushions (Manasek, 1968; Markwald et al., 1975,1977) and all the derivatives of the neural crest (Weston, 1983; Erickson, 1986).

The earliest detectable adhesive interactions between cells in the early mouse embryo occur at the eight-cell stage during compaction, as described above. It is at this point in development that the differentiation of the trophectoderm commences, and the intercellular adhesions that are formed eventually lead to the formation of a mature epithelium. During the peri-implantation period following hatching of the blastocyst from the zona pellucida, the trophectoderm differentiates to form trophoblast (Enders et al., 1981). These cells show considerable protrusive activity and become highly adhesive to the substrate; characteristics that will lead, *in vivo*, to implantation in the uterus, and *in vitro*, to formation of an embryo outgrowth, considered a model for implantation (Mintz,1964; Cole and Paul, 1965; Gwatkin, 1966a, b; Enders et al., 1981).

The formation of outgrowths is dependent upon the presence of suitable culture conditions (Spindle and Pedersen, 1973; Hsu, 1971; 1973). Fetal calf serum and amino acids are required in the medium in order to obtain trophoblast spreading with differentiation of ectoderm and endoderm, and further modifications of the medium are required to obtain development to subsequent stages (Hsu, 1978). An important requirement for trophoblast outgrowth is the presence of a suitable substrate for trophoblast attachment and spreading; embryos cultured on agarose will not form outgrowths even in the presence of serum (Wilson and Jenkinson, 1974). Early

studies showed that matrices of rat-tail collagen would support trophoblast spreading in the absence of serum, although no differentiation of ICM was seen under these conditions (Wilson and Jenkinson, 1974). More recently, it has been reported that substrates of the adhesive molecules fibronectin, laminin, and vitronectin will also support trophoblast spreading in serum-free medium (Armant et al.,1986a, b; Farach et al., 1987).

The ability of blastocysts to attach and spread on these defined ligands suggests the presence of specific cell surface receptors. A complex of cell-matrix adhesion-related glycoproteins of M_r 140,000 has been described in mammalian cells (Knudsen et al., 1981; Damsky et al., 1981; Brown and Juliano, 1985, 1986; Wayner and Carter, 1987) and in avian cells (Neff et al., 1982; Greve and Gottlieb, 1982), that has properties of both a fibronectin and a laminin receptor (Horwitz et al., 1985, 1986; Hasagawa et al., 1985; Akiyama et al., 1986; Hall et al., 1987; Tomaselli et al., 1987; Wayner and Carter, 1987). This complex, recently named integrin in the avian system (Tamkun et al., 1986), has been shown to be a member of a broad family of adhesion receptor complexes interacting with their respective ligands via cell recognition sites containing the tripeptide Arg-Gly-Asp (Pierschbacher et al., 1984b). Among these are the fibronectin, vitronectin, collagen, and fibrinogen receptors (Pytela et al., 1985a, b; see reviews by Ruoslahti and Pierschbacher, 1986, 1987; Hynes, 1987).

The M_r 140,000 avian integrin complex is involved in the migration of neural crest cells (Bronner-Fraser, 1985, 1986) and in neurite outgrowth from sympathetic neurons (Hall et al., 1987). In addition, changes in its distribution correlate with the morphogenetic changes in the developing chick lung (Chen et al., 1986). A role for this receptor complex during implantation of the mouse embryo has also been postulated,

since an antiserum, anti-GP140, which recognizes this receptor complex in mammalian cells (Knudsen et al., 1981; Damsky et al.,1982) blocks attachment of hatched blastocysts to tissue culture plastic in the presence of serum (Richa et al., 1985). Synthetic hexapeptides containing the Arg-Gly-Asp tripeptide will also inhibit trophoblast outgrowth on fibronectin (Armant et al., 1986b; Farach et al., 1987). In Chapter Three of this thesis, I have examined whether blastocysts express the 140 kD adhesion receptor family at a time and location consistent with its proposed function in implantation, and whether blastocysts specifically recognize defined substrates, such as fibronectin, laminin and collagen type IV, that they are likely to encounter during invasion of the uterine stroma.

Chapter One

Summary

The polarization hypothesis of early mouse development proposes that the divergence of the trophectoderm and inner cell mass of the blastocyst originates from the structural polarization of the 8-cell blastomeres during compaction, followed by segregation of the different regions of the 8-cell blastomere into different populations of 16-cell blastomeres by delaminatory division (Johnson et al., 1981). Polarization results in the asymmetric distribution of both cell surface and intracellular components of the 8-cell blastomere (Reeve and Ziomek, 1981; Johnson and Ziomek, 1982; Fleming and Pickering, 1985; reviewed in Johnson and Maro, 1986). Division of polarized 8-cell blastomeres results in the formation of two populations of 16-cell blastomeres that differ in position and in phenotype; outer cells of the 16-cell morula are larger and polarized, whereas inner cells are smaller and non-polarized (reviewed in Johnson and Maro, 1986). The cinemicrographic analysis of the early cleavage stages of the mouse embryo presented in this chapter has provided new information on the process of cell allocation during fourth cleavage. Observations on the timing of cleavage divisions and length of cell-cycle have confirmed results obtained with isolated blastomeres (Kelly et al., 1978) showing that cleavage asynchrony depends mostly on random chance, and demonstrates that it is intrinsic to the blastomeres. Variability within and between embryos is not confined to the timing of cleavage divisions, but is also observed in the timing of cell flattening associated with compaction. Cinemicrographic analysis has made possible the determination of the frequencies of the different types of cleavage divisions during fourth cleavage, and has revealed that there are three, rather than two, major planes of division. The frequencies observed are consistent with the polarization hypothesis, and support the notion that inner cells of the morula are generated by delaminatory division during fourth cleavage. The fact that the type of division observed for any blastomere does

not correlate with either its lineage from the 2- or the 4-cell stage or with the division order during fourth cleavage suggests that mechanical forces or packing patterns within the 9- to 16-cell embryo also play some role in determining blastomere fate.

Materials and Methods

Embryo Culture

Female ICR mice (12 weeks old, Harlin) were superovulated by injection with 5 IU of pregnant mare's serum (Teikoku Hormone Mfg. Co., Japan) followed after 48 hours by an injection of 2.5 IU of human chorionic gonadotropin (hCG, Sigma) and were then caged with ICR males, and inspected the next morning for the presence of a vaginal plug, indicating insemination. Embryos were collected at the 2-cell stage (48 hours post-hCG) by flushing them from the oviducts using modified embryo culture medium (Biggers et al., 1971; Spindle, 1980), and then put into culture for cinemicrography as described below.

Cinemicrography

Embryos were placed in a microdrop of culture medium under oil in a Falcon 35 mm tissue culture dish (#3001) that had been equilibrated at 37° C for four hours. The cover of the dish was inverted and two holes bored into opposite sides; one a pinhole, the other large enough to accept a syringe tip. The cover was then sealed onto the dish using high vacuum grease (Dow Corning). A piece of tubing was inserted into the large hole in the cover, and attached at at the other end to a tank containing 5% CO2 in air. The flow of gas was adjusted to a level that did not disturb the surface of the oil.

The chamber was then placed into a stage heater (Steier, 1975) controlled by a proportional temperature controller (Yellow Springs, Model 72) on the stage of a Nikon Diaphot inverted microscope outfitted with Hoffman Modulation Contrast optics.

Comparisons between embryos allowed to develop in this stage heater and those from the same batch left in the incubator revealed no differences in rate of development or the percent reaching the blastocyst stage. The embryos were filmed using a 10X or 20X objective, and their development recorded on either Kodak Plus-X reversal or Tri-X reversal film with a Bolex H-16 camera controlled by a Nikon CFX intervalometer. Frames were taken at one-minute intervals. In most cases a green filter was used, which was described by others as beneficial to development (Mulnard,1967), but under the above-described conditions development was unaffected by the presence or absence of the filter. The resulting films were analyzed using a film analysis projector (NAC, Model 16C).

Results

Cleavage stage embryos were filmed beginning at the late two-cell stage (50-52 hours post-hCG), and continuing for at least the next 40-48 hours (to the early blastocyst or 32-cell stage), and sometimes for the next 72 hours (to the expanded blastocyst or 64-cell stage). In the following sections, I will refer to the cytokinetic phase of each cycle as a particular cleavage, and the period between divisions as a particular stage. For example, the division in which the embryo goes from two to four cells will be referred to as second cleavage, and the time between the end of second cleavage and the start of third cleavage will be referred to as the four-cell stage. Subdivisions of these two periods could not be made on the basis of cinemicrographic observations. Observations on the timing of cleavage stages and on planes of divisions were made only up to the beginning of the fifth cleavage division (16 to 32 cell division) because after this it became impossible to accurately determine either the start or finish of the succeeding cleavages, or the division planes of the individual blastomeres.

Timing of Stages and Cleavage Divisions

Cleavage was asynchronous between blastomeres in all cleavage divisions observed. In some cases, the 2-cell blastomeres divided synchronously, and certain blastomeres divided nearly synchronously during third and fourth cleavage, but these events were rare and appeared to be due to chance. The amount of time spent in division varied greatly between embryos for each particular cleavage and increased with each succeeding cleavage. The distribution of long and short division times was random between embryos as well as between different cleavage divisions in any individual embryo. Figure 1-1 illustrates the mean time spent in division during second, third, and fourth cleavage. The time intervals between the division of each of

the four blastomeres during fourth cleavage are shown in Figure 1-2. These intervals are important in the interpretation of experiments done to examine the relative contribution of inner descendants of the earlier- dividing 4-cell blastomere versus the later-dividing 4-cell blastomere (see Discussion).

The mean cell-cycle times of blastomeres were similar for the 4-cell and the 8-cell stages (Figures 1-3 and 1-4). At each stage, there were two categories of cell cycle times, those of the earlier-dividing daughters of the 2- or 4-cell blastomeres, and those of the later-dividing daughters. Within each of these categories the cell cycle times were similar. Moreover, the mean cell cycle time of the daughters of the earlier-dividing blastomeres were comparable to those of the later-dividing blastomeres, showing that there is no heredity of cell-cycle time from the 2- to the 8-cell stage. This fact also demonstrates that the length of the cell cycle of a blastomere at any of these stages does not bestow upon it any special qualities, and it is not a basis for differentiating that blastomere from all the others.

The amount of time spent between cleavages, in each particular stage, also varied between embryos, and decreased from an average of 8.8 hours for the 4-cell stage to an average of 5.9 hours for the sixteen-cell stage (Figure 1-5). This observation simply reflects the fact that the time spent in division increases with each succeeding cleavage while the cell cycle times of the blastomeres are not changing. Therefore, the same amount of time passes between the first division of one cleavage and the first division of the next, but more of the intervening time is spent with some cells in division. Following compaction, during the eight-cell stage, the preparation of the blastomeres for fourth cleavage can be clearly observed. Rounding up and decompaction of each of the flattened 8-cell blastomeres occurs roughly 30 minutes before it divides. Usually, one or two blastomeres out of 8 would round up at a time

throughout fourth cleavage, but sometimes most or all of the blastomeres would round up at the very beginning of fourth cleavage, causing the entire embryo to decompact. In these latter cases the divisions of the decompacted blastomeres were still asynchronous, although the intervals between the succeeding divisions were only a few minutes.

Observations on Compaction

The morphogenetic process of compaction involves the flattening of blastomeres upon one another to form a roughly spherical embryo. Compaction occurs at the eight-cell stage concomitant with the first formation of gap and focal tight junctions, and represents the epithelialization of the embryo (Lewis and Wright, 1935; Ducibella and Anderson, 1975; Magnuson et al., 1977; McLachlin et al., 1983). Observed cinemicrographically, the process of compaction is not a quick and discrete event but is slow, gradual, and progresses throughout the 8-cell stage. The time of initiation of flattening, as well as the degree of blastomere flattening prior to fourth cleavage, varied between embryos; many were "compacted" at the four-cell stage (Figure 1-6a), and, less often, others remained uncompacted until after fourth cleavage, during the 16-cell stage (Figure 1-6c). The timing of blastomere flattening was unrelated to viability; both those embryos that flattened early and those that flattened late formed normal blastocysts (Figure 1-6b and d). In the embryos that were observed to undergo flattening at the 8-cell stage, the degree to which the blastomeres flattened upon one another prior to fourth cleavage also differed between embryos (Figure 1-6e,g,i,k). This may represent variability in the rate of compaction, because the degree of blastomere flattening was similar for all embryos by the end of the 16-cell stage (Figure 1-6f,h,j,l). In summary, it appears that there is no absolute connection between cell cycle number and compaction. The process is

gradual, and may begin as early as the 4-cell stage or as late as the 16-cell stage, and is always complete, in normal development, by the end of the 16-cell stage.

Division Planes of 8-cell Blastomeres

The division planes of 8-cell blastomeres during fourth cleavage were closely examined in an attempt to determine the relative occurrence of periclinal (parallel to the external surface of the blastomere) and anticlinal (perpendicular the external surface of the blastomere) divisions in the intact embryo (See Figure 1-7 for illustration.). Previous studies have used a different terminology, in which periclinal divisions are termed differentiative divisions, and anticlinal divisions are termed conservative divisions (Johnson and Ziomek, 1981; Ziomek and Johnson, 1982; reviewed in Johnson and Maro, 1986). Use of the term "conservative" to mean that anticlinal division produces two like blastomeres is appropriate. Likewise, use of the term "differentiative" to mean that periclinal division produces two unlike blastomeres, no matter how transient or ephemeral their differences, is also appropriate. However, the word "differentiative" may imply that determinative and differentiative events have occurred, restricting the potency of the blastomeres which is not the case (Hillman et al., 1972; Ziomek and Johnson, 1982; Ziomek et al., 1982; Johnson and Ziomek, 1983). Therefore, in this thesis I adopt the terminology of Wilson, (1896) in describing the orientation of the division planes of 8-cell blastomeres, in order to avoid making any assumptions about the positional fate or state of determination of the daughter cells.

In 22 embryos in the 8 films examined, the fourth cleavage division planes for all eight of the blastomeres could be clearly distinguished. The analysis of division plane orientations was done for each of these embryos on three separate occasions and without reference to results from any previous determination. There was a 5%

discrepancy between successive evaluations, and all of these were re-examined and a final determination made.

It became clear after observing several embryos that there were not just two different planes along which these blastomeres would cleave, but there were in fact three. The third plane lies roughly at a 45o angle to the other two, and I have called this type of division an oblique division (Figure 1-8). The geometry is intermediate to that of the periclinal and anticlinal divisions, and so cannot be classified as strictly one or the other. The structural characteristics of the daughter cells (i.e. number and distribution of surface microvilli, or distribution of internal cellular components) of this type of division cannot be determined by cinemicrography, but may be important to their positional fate in the morula. This point will be taken up in the Discussion.

The mean numbers of each type of division per the eight divisions that comprise fourth cleavage are shown in Figure 1-9. The mean numbers of periclinal divisions (3.2) and anticlinal divisions (2.8) per embryo during fourth cleavage were not significantly different from one another (Student's t test). Periclinal divisions were significantly more frequent than oblique divisions at the 0.5% level (Student's t test) and anticlinal divisions were significantly more frequent than oblique divisions at the 1% level (Student's t test). However, there was a wide variation in the relative number of each of the three types of divisions observed in individual embryos . The number of periclinal divisions observed per embryo ranged from 0 to 7, the number of anticlinal divisions from 1 to 7, and the number of oblique divisions from 0 to 4. No embryos were observed to have only one type of division during fourth cleavage.

Cell Lineage and Division Plane during Fourth Cleavage

A number of studies have reported that the descendants of the earlier-dividing 2-cell blastomere contribute preferentially to the ICM at the blastocyst stage (Kelly et al., 1978; Surani and Barton, 1982), and recently Garbutt et al. (1987) reported that earlier-dividing 4-cell blastomeres could contribute more descendants to the inner cells of the morula than later-dividing 4-cell blastomeres. A stated inference of this last result was that the 8-cell descendants of the earlier-dividing 4-cell blastomeres were more likely to undergo periclinal divisions as opposed to anticlinal divisions (Garbutt et al., 1987). To determine whether this was true, another analysis was performed on the 22 embryos described above. The embryos were traced at the 4-cell stage, the blastomeres were labelled according to the order of their division (1 to 4), and their descendants examined as to their order of division and their division plane during fourth cleavage. As previously, this analysis was performed on three separate occasions for each embryo, and discrepancies between successive evaluations (approximately 1%) were re-examined and a final determination made. The results of this analysis are shown in Figures 1-10 to 1-15.

For both third and fourth cleavage, the division order of the blastomeres was found to be correlated with the division order of their progenitors at the previous stage (Figures 1-10 to 1-12). These correlations reflect the similarity of cell cycle time between blastomeres; those that are descendants of the earlier-dividing 2-cell blastomere tend to stay ahead of those that are descendants of the later-dividing 2-cell blastomere simply because they have a head start. When division order at the 8-cell stage is compared with the lineage from the 2-cell stage, it is clear that the correlation tends towards randomization with successive cleavage divisions (Figure 1-12).

The type of division (periclinal, anticlinal, or oblique) undergone by each blastomere during fourth cleavage was then compared with the lineage of that blastomere from both the 2-cell stage and the 4-cell stage (Figures 1-13 to 1-15). When these results were analyzed statistically, they showed that cleavage type is related neither to lineage from either the 2- or the 4-cell stage, nor to fourth cleavage division order. The distribution of the three division types is statistically similar between the 8-cell descendants of AB and CD blastomeres (c^2 test on a 2X3 contingency table, $p<.50$; Figure 1-13) and between the 8-cell descendants of the first-, second-, third-, and fourth-dividing 4-cell blastomeres (c^2 test on a 4X3 contingency table, $p<.20$; Figure 1-14). The distribution of the three division types was also similar for all eight divisions during fourth cleavage (c^2 test on an 8X3 contingency table, $p<.70$; Figure 1-15).

Discussion

Timing During Cleavage Stages

The use of cinemicrography allows a very accurate determination of the timing of various events during preimplantation mouse development. The results described above illustrate several interesting features of timing. First, there is enormous variability in the timing of cleavage divisions both between embryos and between individual blastomeres within an embryo. This has been noted in previous cinemicrographic studies on mouse embryos (Cassini,1962; Borghese and Cassini, 1963; Mulnard, 1967) as well as on other mammals (Lewis and Gregory, 1929; Lewis and Hartman, 1931). The mean times for the various cleavage divisions and stages found here agree with those found by others for dissociated blastomeres (Kelly et al., 1978), indicating that timing of cleavage is probably intrinsic to the blastomeres and is not regulated among blastomeres. The asynchrony of division between blastomeres results in a systematic increase in the amount of time the embryo spends in division for each succeeding cleavage division, and, since there is no change in the mean cell-cycle time, leads to a systematic decrease in the amount of time spent between divisions. This pattern was also described by Mulnard (1967).

The timing of cell flattening is also variable. This event is usually associated with the compaction of the embryo during the 8-cell stage (Ducibella and Anderson, 1975). However, the films demonstrated that the blastomeres of 4-cell embryos were frequently highly flattened on one another, similar to what is seen in 8-cell embryos (Figure 1-6a). Less frequently, the blastomeres of 8-cell embryos remained rounded until fourth cleavage, or showed variable degrees of flattening prior to fourth cleavage (Figure 1-6). This variability did not affect the embryo's viability; both those that were flattened during the 4-cell stage and those that did not flatten during

the 8-cell stage formed morphologically normal blastocysts (Figure 1-6). It has been suggested that onset of cell flattening occurs when a negative regulating factor is inactivated at the beginning of the 8-cell stage (Levy et al., 1986). If so, the variation in the time of onset of cell-flattening observed here reflects variability in the time of inactivation of this negative regulating factor, and demonstrates that the degree of flattening achieved by the end of the 8-cell stage is not critical to further development. In fact, in studies where cell flattening at the 8-cell stage was inhibited artificially, both junction formation and polarization of the blastomeres still took place, indicating that these other major features of compaction are not dependent on cell flattening, and consistent with the idea that variable degrees of flattening at the 8-cell stage are compatible with normal development (Ziomek and Johnson, 1980; McLachlin et al., 1983; Johnson et al., 1986).

Division Order and Lineage

Tracing the lineage of both 4-cell blastomeres and 8-cell blastomeres demonstrates that the division order of the blastomeres during any particular division is correlated with the division order of the parent blastomeres during the previous cleavage division, and to a certain extent with the division order of the progenitors of two divisions previous. These observations agree with the findings of Kelly et al. (1978) on dissociated blastomeres. This association between the division order at one stage and that of the next is due to their descent from either the earlier- or later-dividing blastomere at the 2-cell stage. Those daughters of the earlier-dividing 2-cell blastomere get a head start on those of the later-dividing 2-cell blastomere, and thus tend to stay ahead. By the 8-cell stage, however, the pattern has randomized considerably, and only the first and last divisions show a very strong correlation.

Types of Cleavages during Fourth Cleavage

The polarization hypothesis, as put forward by Johnson and co-workers, proposes that differences in apical and basal regions of 8-cell stage blastomeres are maintained during fourth cleavage and result in the generation of two populations of 16-cell blastomeres with different morphological and behavioral characteristics (Johnson et al., 1981; Johnson, 1981; Johnson and Ziomek, 1981; Ziomek and Johnson, 1981; reviewed in Johnson, 1985). The inner cells of the morula are thus thought to be generated by delaminatory division of 8-cell blastomeres, and the relative number of inner and outer cells in the 16-cell morula is thought to depend on the proportion of two types of divisions during fourth cleavage, one parallel (periclinal) and one perpendicular (anticlinal) to the axis of radial polarity (Johnson and Ziomek, 1981; Johnson, 1981; Johnson, 1985; Garbutt et al., 1987; Fleming, 1987). Periclinal (or differentiative) divisions would result in the partition of the apical and basal regions of the blastomere, and would thus generate outer-inner pairs of sister blastomeres at the 16-cell stage. Anticlinal (or conservative) divisions, on the other hand, would partition the 8-cell blastomere along the axis of polarity, resulting in two structurally similar daughter cells, and would therefore generate outer-outer pairs of blastomeres in the morula. Two lines of evidence support these assumptions. First, a pair of labelled 8-cell blastomeres gives rise to a maximum of two, and a minimum of no inside cells at the 16-cell stage (Pedersen et al., 1986; Fleming, 1987; Garbutt et al., 1987). This means that each 8-cell blastomere has at least one outer descendant in the morula, and precludes the idea that inner cells are randomly recruited (Pedersen et al., 1986; Fleming, 1987; Garbutt et al., 1987). Second, the outer and inner cells of the 16-cell embryo each have a distinctive surface-binding pattern for the lectin Con-A, which corresponds to a pattern of surface microvillar distribution (Handyside, 1980; Reeve and Ziomek, 1981). These patterns are consistent with the idea that periclinal divisions of the 8-cell blastomere

generate outer-inner pairs, and anticlinal divisions outer-outer pairs (Johnson and Ziomek, 1981; Fleming and George, 1986; Fleming, 1987; reviewed in Johnson, 1985).

The inner cells of the morula can still develop the morphology of outer cells, however, and can then form trophectoderm (the usual fate of outer cells) (Handyside, 1978; Hogan and Tilly, 1978; Spindle, 1978; Ziomek and Johnson, 1982; Johnson and Ziomek, 1983). Since the division planes of 8-cell blastomeres have never before been examined directly in the intact embryo, the orientation of the division planes during fourth cleavage has only been assumed to be the determinative factor in allocation of inner and outer cells in the morula. The frequency of different division plane orientations has been examined in small, pooled populations of isolated 8-cell blastomeres (Johnson and Ziomek, 1981) but is not representative of the situation for blastomeres that are in contact with others (Johnson and Ziomek, 1983; Johnson and Maro, 1986). The frequencies of anticlinal and periclinal divisions during fourth cleavage have also been inferred from examining the positions or phenotypes of the daughter 16-cell blastomeres, either in the intact embryo (Balakier and Pedersen, 1982; Pedersen et al., 1986; Fleming, 1987), reaggregated embryos (Garbutt et al., 1987), or dissociated blastomeres (Johnson and Ziomek, 1981; Ziomek and Johnson, 1982). However, this method does not provide any direct information on the orientations of division planes, but rather allows determination of the qualitative results of those cleavages; namely generation of outer-outer or outer-inner pairs of 16-cell blastomeres (or polar-polar and polar-apolar pairs by phenotype).

In the experiments described here, the use of cinemicrography made possible the determination of the actual frequencies of each type of division in the intact, unmanipulated embryo, and revealed that 8-cell blastomeres will divide along not only

anticlinal and periclinal planes, but will also divide along an intermediate, or oblique, plane. In fact, the difficulty encountered in classifing some divisions may indicate that 8-cell blastomeres can divide along any plane in a continuous fashion, but that the resolution of the cinemicrographic technique is not high enough to distinguish more than three - clearly periclinal, clearly anticlinal, and neither of the above (oblique). That the 8-cell blastomere can divide along more than two planes was noted for dissociated blastomeres (Johnson and Ziomek, 1981). However, in that study, the oblique divisions were categorized along with the periclinal divisions as being distinct from anticlinal divisions. This may be an inappropriate classification. While it is true that in the oblique division the cleavage plane crosses the axis of polarity of the 8-cell blastomere, it does so in an intermediate manner relative to the periclinal division, and would segregate the apical and basal regions of the blastomere differently than either the anticlinal or periclinal divisions. Since the structural characteristics of the daughter cells of oblique divisions could not be determined, it is not clear how the apical pole of microvilli is partitioned between the two daughters, or indeed, whether the partitioning is always the same. The critical parameter determining cell fate may be whether or not oblique divisions include part of the apex of the polarized cell in both blastomeres. This is an unresolved issue at this time.

On average, there are equal numbers of anticlinal and periclinal divisions during fourth cleavage, in addition to a smaller number of oblique divisions (Figure 1-9). The ultimate fate of the daughter blastomeres could not be determined from cinemicrographic observations, however, some possible outcomes can be considered. If it is assumed that periclinal divisions give rise only to outer-inner pairs and anticlinal divisions only to outer-outer pairs, then the average number of inner cells at the 16-cell stage should be at least 3.2. If, in addition, oblique divisions then give rise only to outer-outer pairs, the average number of inner cells should be no more than

the average number of periclinal divisions, or 3.2. In this case, the frequency of generating outer-outer pairs of sister blastomeres during fourth cleavage should be greater than that of generating outer-inner pairs. A second possibility is that oblique divisions give rise to variable numbers of outer-inner and outer-outer pairs. In this case the number of inner cells should be between 3.2 and 5.2, and there should be an equal chance of generating an outer-outer or outer-inner pair for any 8-cell blastomere. A third possibility is that oblique divisions give rise only to outer-inner pairs, as predicted from examinations of isolated blastomeres (Johnson and Ziomek, 1981). In this case, the average number of inner cells should be 5.2, and there should be a greater frequency of outer-inner pairs than outer-outer pairs.

Previous studies have determined the relative frequencies of outer-outer and outer-inner pairs of sister blastomeres at the 16-cell stage (Gearhart et al., 1982; Balakier and Pedersen, 1982; Pedersen et al., 1986), as well as the average total number of inside cells at the 16-cell stage (Fleming, 1987; Garbutt et al., 1987). Labelling the outer cells of the morula with fluorescent beads, disaggregating the embryo, and counting the numbers of labelled and unlabelled blastomeres gave an average of 5.2 inner cells (Fleming, 1987). The same procedure done on morulae developing from reaggregated 8-cell embryos came up with several estimates, all between 4.0 and 5.0, and all statistically similar (Garbutt et al., 1987). Injection of lineage tracers into 8-cell blastomeres, followed by inspection of the position of daughter cells of the injected blastomere in the morula has shown that there is an equal chance of generating an outer-outer or outer-inner pair of sister blastomeres at fourth cleavage (Gearhart et al., 1982; Balakier and Pedersen, 1982; Pedersen et al., 1986). The large range in the number of inside cells between embryos (2 to 7) found by Fleming (1987) also correlates with the large range in the number of periclinal divisions observed per embryo here (none to 7). The fact that the lowest number of

inside cells seen was two (Fleming, 1987) and the lowest number of periclinal divisions was none supports the concept of allocation of inner cells by oblique divisions as well as periclinal divisions. Taken together, all of these results are consistent with the second case presented above; that periclinal divisions give rise only to outer-inner pairs, anticlinal divisions to outer-outer pairs, and oblique divisions to either outer-outer or outer-inner pairs.

The average number of inner cells found in several other studies (6-7, Handyside, 1981; 7, Johnson and Ziomek, 1981; 5.2, Fleming, 1987) also supports the third possibility described above, that oblique divisions give rise only to outer-inner pairs. However, the frequency of outer-inner pairs in the morula is not high enough to account for all these divisions (Gearhart et al., 1982; Balakier and Pedersen, 1982; Pedersen et al., 1986). It may be, as suggested by Fleming (1987), that there are strain differences in allocation of inner cells; if so, it would be interesting to examine the relative frequencies of the different cleavage plane orientations in those strains that differ greatly in the average number of inner cells.

The variable allocation of the apical region of the 8-cell blastomere to the daughter 16-cell blastomeres by an oblique division plane may be the reason that oblique divisions can give rise to both outer-outer and outer-inner daughter blastomeres. It has been assumed for anticlinal and periclinal divisions that the segregation of the apical and basal regions of the blastomere results in the generation of an outer and an inner blastomere, whereas equal partitioning of apical and basal regions results in the generation of two outer blastomeres. In both of these cases, the outer cells of the morula contain all or some of the apical region of the 8-cell blastomere, and may contain some of the basal region. In contrast, the inner cells of the morula contain none of the apical region, only the basal region. This would suggest

that certain properties of the apical region of the 8-cell blastomere causes the daughter blastomeres receiving all or some of it to become an outer cell. These properties could exist in a graded fashion; the greatest amount at the apex of the cell, then decreasing towards the basal region, and the relative amount allocated to a daughter cell determining whether or not it becomes an outer cell. Oblique divisions would then apportion variable amounts of these apical properties to the daughter cells depending on the angle of division. Alternatively, the apical determining properties could be localized in a discontinuous fashion, all at the apical pole of the cell. In this case, the oblique division may either cut through this region, or narrowly miss it, resulting either in both daughter cells containing some apical region, or one with and one without. In both cases, the oblique division would give rise variably to both outer-outer and outer-inner blastomeres at the 16-cell stage.

The existence of oblique cleavage planes may help to explain some other observations on morulae. Both Soltynska (1982) and Pedersen et al. (1986) noted the presence of some 16-cell blastomeres that were mostly enclosed, but had a small area of surface membrane exposed to the exterior of the embryo. These may be daughter cells of an oblique division that comprised more of the basal region of the cell and only a small portion of the apical region. Fleming and George (1986) found a small number of cells that were categorized as apolar (or inner) yet would label sparsely with fluorescent latex beads; these may also represent partially enclosed blastomeres that have only a small amount of surface area available for bead binding.

Lineage and Division Type

Previous studies have shown that the earlier dividing 2-cell blastomere (AB) contributes more descendants to the inner-cell mass of the blastocyst than does the later dividing 2-cell blastomere (CD) (Kelly et al., 1978; Spindle, 1982; Surani and Barton, 1984). This was extended to show that earlier-dividing 4-cell blastomeres could contribute more descendants to the inner cell population of the morula than later-dividing 4-cell blastomeres (Garbutt et al., 1987). The stated inference of these results is that the earlier dividing 8-cell blastomeres undergo periclinal division more frequently than do the later-dividing 8-cell blastomeres (Garbutt et al., 1987). My cinemicrographic analysis, however, reveals that the distribution of the various division types is similar for all combinations tested. The descendants of both AB and CD blastomeres have similar frequencies of periclinal, anticlinal and oblique divisions during fourth cleavage (Figure 1-13). The distributions of the three division types is also similar for the descendants of all the 4-cell blastomeres, regardless of third cleavage division order, and for all the 8-cell blastomeres regardless of fourth cleavage division order (Figures 1-15).

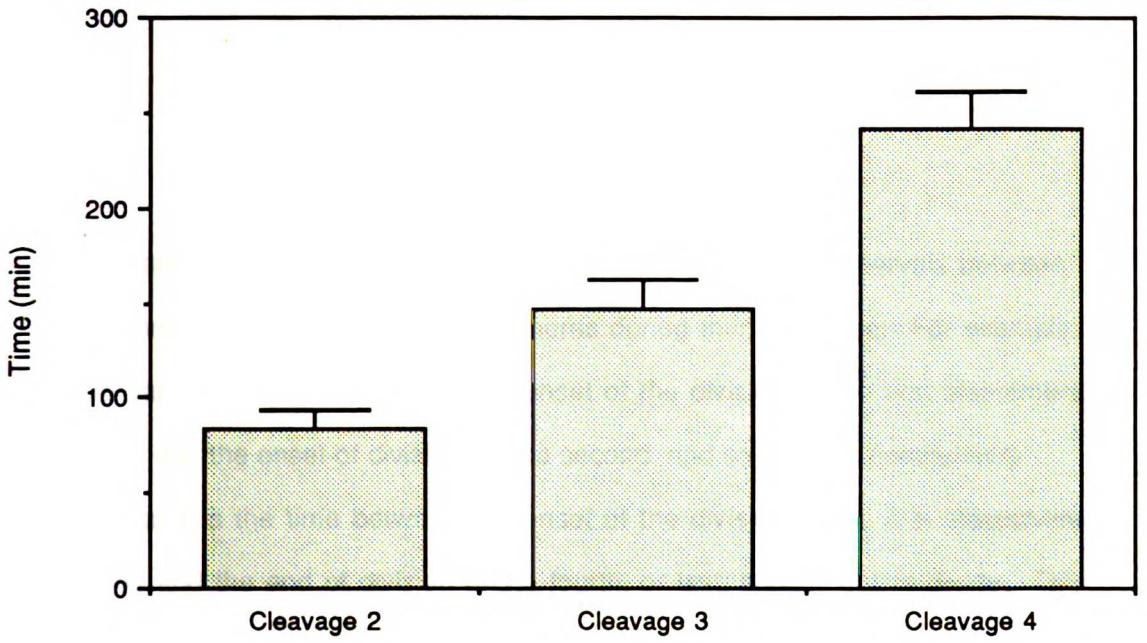
These results seem somewhat paradoxical, inasmuch as several studies show an influence of division order on the extent of allocation to the inner cell population (Kelly et al., 1978; Spindle, 1982; Surani and Barton, 1982, Garbutt et al., 1987), and, as described above, the mean numbers of periclinal and oblique divisions are very consistent with the mean number of inner cells seen in the 16-cell stage embryo. However, an important point should be made about the experimental procedure used to examine the relationship between division order and allocation in the studies cited above. Garbutt et al. (1987) used reconstructed 8-cell embryos consisting of two labelled blastomeres and six unlabelled blastomeres. In each case, the pair of labelled blastomeres was either four hours younger or four hours older than the other six

blastomeres. My cinemicrographic observations on normal embryos reveal that this type of construct is entirely artificial. The average amount of time between the first and second divisions of third cleavage is 34 minutes, the average amount of time between the second and third divisions of third cleavage is 50 minutes, and the average total time spent in third cleavage is 143 minutes (see Figures 1-1 and 1-2). In no case was any embryo observed to have an interval of 240 minutes between any of the divisions of third cleavage. The advantage gained by the earlier-dividing blastomeres of these reconstructed embryos is thus unlikely to play a role in normal development. In some of the other studies cited above (Spindle, 1982; Surani and Barton, 1984) the blastomeres used to create the reconstructed embryos were at different stages (i.e. 2-cell and 4-cell blastomeres, or 4-cell and 8-cell blastomeres), and thus could be even further apart than four hours, especially given the asynchrony between embryos. In the one study where the reconstructed embryos comprised only the original blastomeres from one embryo (Kelly et al., 1978), the observed advantage at the blastocyst stage of earlier-dividing blastomeres was actually quite small in terms of numbers of cells (2-3; Kelly et al., 1978; Surani and Barton, 1982; reviewed in Pedersen, 1986). Any effect at the morula stage is probably even smaller, given that inner cell allocation continues during fifth cleavage (Pedersen et al., 1986; Fleming, 1987)

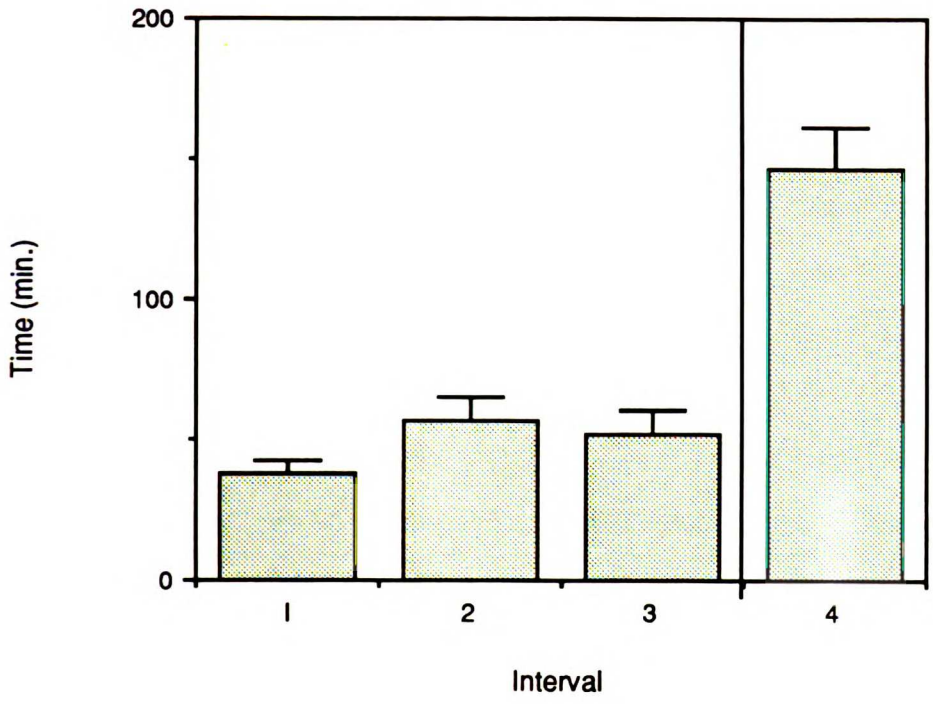
An hypothesis that fits all of the above data is that the oblique divisions that occur early in fourth cleavage give rise more frequently to outer-inner pairs than to outer-outer pairs, whereas oblique divisions that occur late in fourth cleavage give rise more frequently to outer-outer pairs. As a result, there would be a slight bias towards generation of outer-inner pairs during the first half of fourth cleavage which would coincide with the bias for 8-cell descendants of earlier-dividing progenitors (at either the 2-cell or 4-cell stage) to divide earlier. This, in turn, would give an

advantage to the earlier dividing blastomeres in terms of allocation of descendants to the ICM. This putative bias need not be very large; as discussed above, the effect of division order on allocation at the morula stage in the intact embryo is likely to be quite small. Garbutt et al. (1987) observed that the factor operating during the 8-cell stage that favored allocation of descendants of the earlier-dividing blastomere to the inner cell population was the time of cell flattening. The timing of cell flattening or perhaps the packing arrangement of the flattened blastomeres (Graham and Deussen, 1978) may regulate the position of the oblique cleavage planes, and thus, perhaps, the positional fate of the daughter cells.

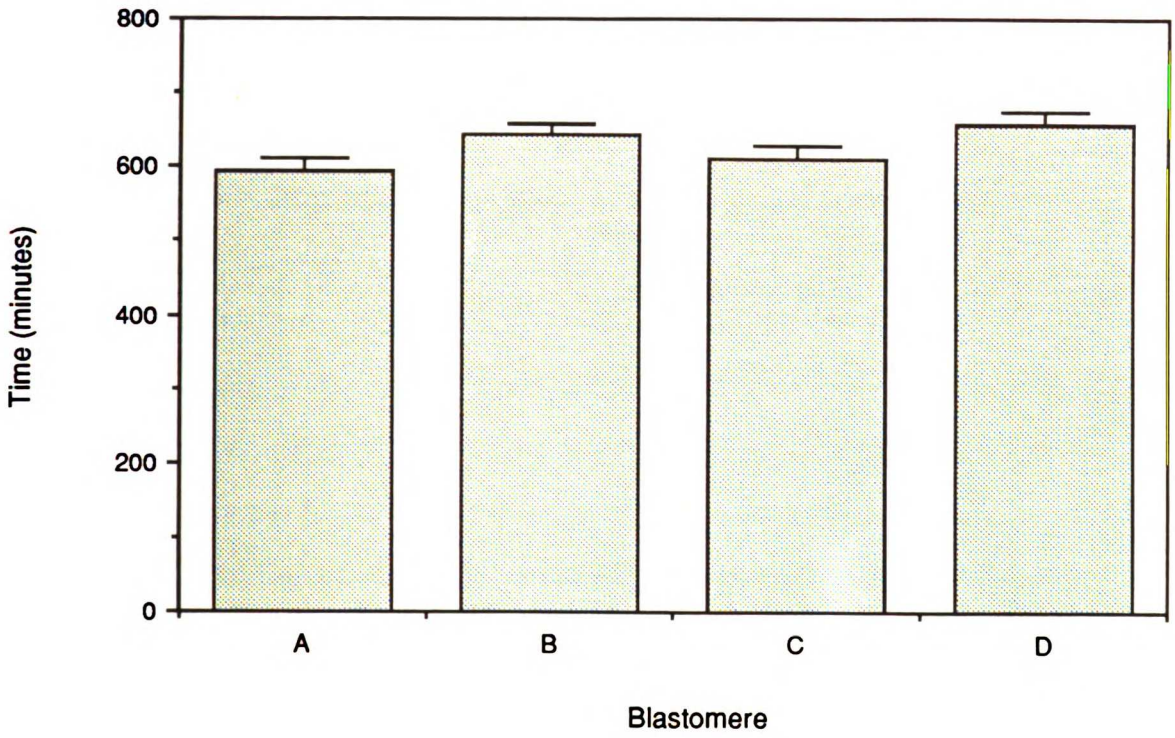
1-1). The graph shows the mean time in minutes spent in division for second, third and fourth cleavages. Time spent in division is defined as the interval between the onset of division of the first blastomere to divide, and the end of division of the last blastomere to divide. The sample sizes were 27 for second cleavage, 38 for third cleavage, and 26 for fourth cleavage. All values are shown ± S.E.M.


300
200
100
0
Time (min)
Cleavage 2
Cleavage 3
Cleavage 4

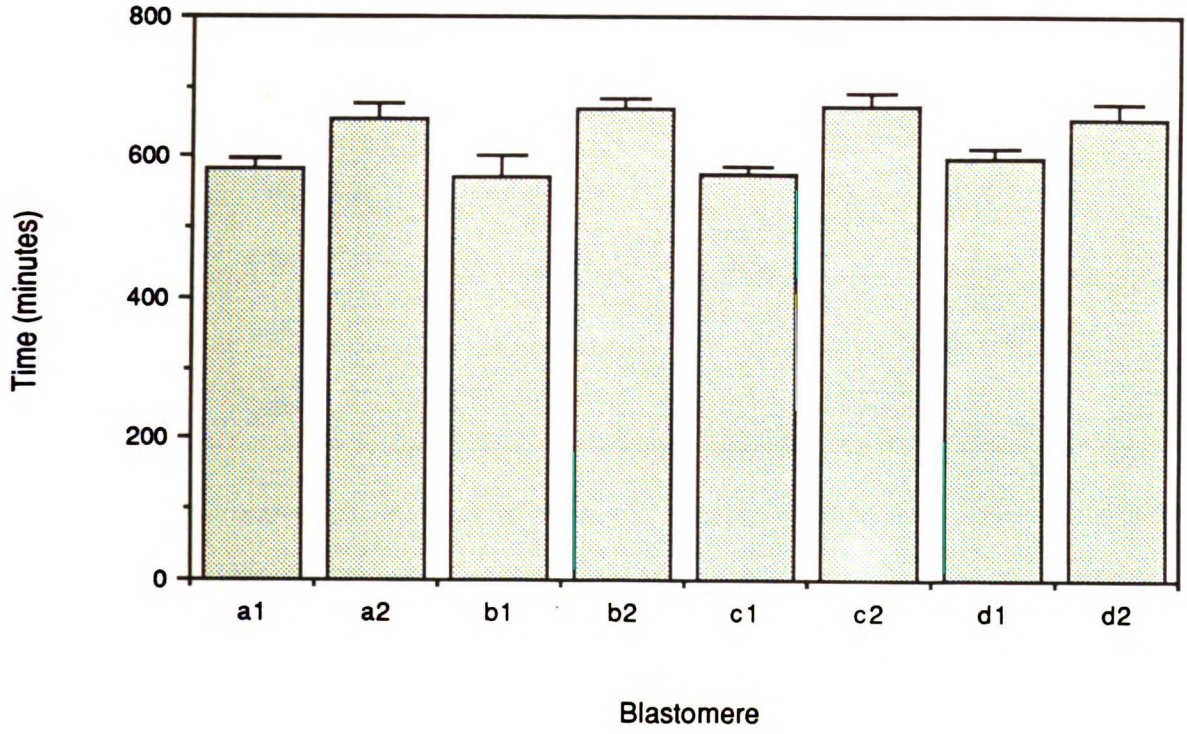
1-2). The graph shows the mean length of time in minutes of the intervals between the divisions of each of the four blastomeres during third cleavage. For example, interval 1 is the time between the onset of the division of the first blastomere to divide and the onset of division of the second, and so on. For comparison, interval 4 is the time between the onset of the division of the first blastomere to divide and the end of division of the fourth, or last, blastomere to divide. This interval is identical to the mean time spent in third cleavage, as illustrated in Figure 1-1. The sample size for these determinations was 38, and each value is shown ± S.E.M.


200
100
0
Time (min.)
1
2
3
4
Interval

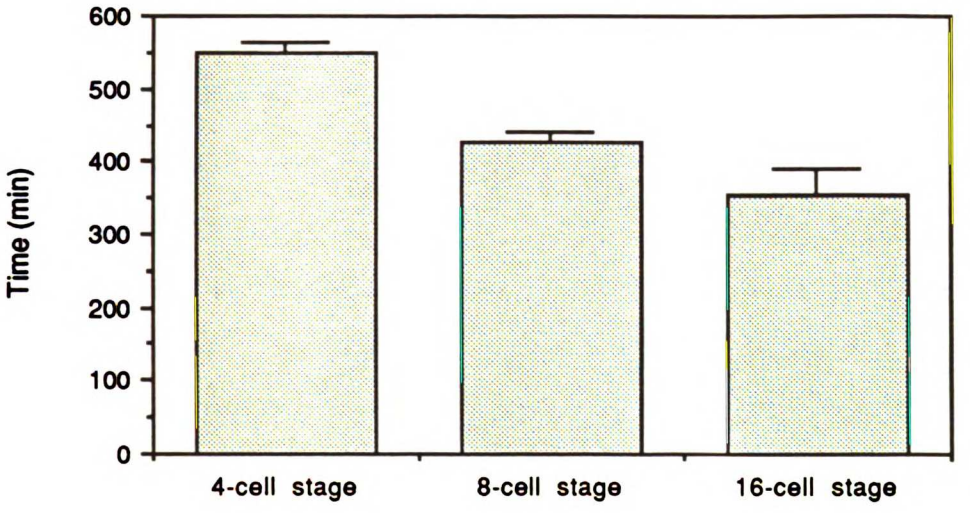
1-3). The graph shows the mean cell cycle time for each of the four blastomeres at the 4-cell stage. Each blastomere is identified both with respect to its progenitor at the 2-cell stage and to its division order relative to its sister blastomere. The earlier-dividing 2-cell blastomere is called "AB", whereas the later dividing 2-cell blastomere is called "CD" The two daughter blastomeres of AB are designated "A" and "B", with the first of the two to divide being A and the second to divide being B. The two daughter blastomeres of CD are designated "C" and "D", with the first to divide being C and the second to divide being D. The sample size was 19, and all values are shown ± S.E.M.


800
600
400
200
0
Time (minutes)
A
B
C
D
Blastomere

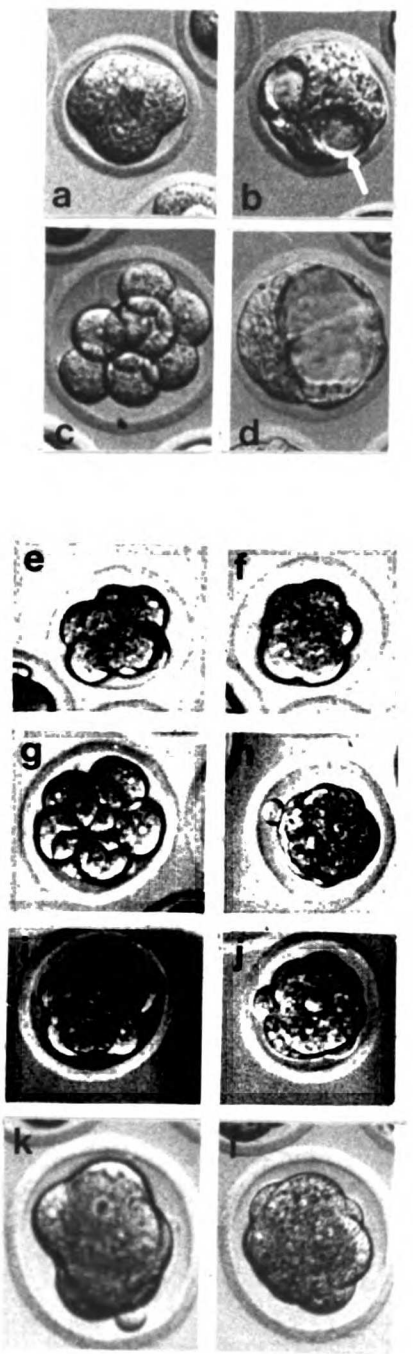
1-4). The graph shows the mean cell cycle times of each of the 8-cell blastomeres. As for Figure 1-3, each blastomere was identified by its lineage from the 2- and 4-cell stages, and by its division order relative to its sister blastomere. As described in Figure 1-3, The earlier-dividing 2-cell blastomere is called "AB", whereas the later dividing 2-cell blastomere is called "CD" The two daughter blastomeres of AB are designated "A" and "B", with the first of the two to divide being A and the second to divide being B. The two daughter blastomeres of CD are designated "C" and "D", with the first to divide being C and the second to divide being D. At the 8-cell stage, the daughter blastomeres of A are designated **a**, those of B are **b**, those of C are **c**, and those of D are **d**. The number 1 signifies the earlier-dividing daughter, while the number 2 signifies the later-dividing daughter. Thus, **a**1 is the earlier-dividing 8-cell daughter of A, and **a**2 is the later-dividing 8-cell daughter of A. The sample size for these determinations was 19, and all values are shown ± S.E.M.


800
600
400
200
0
Time (minutes)
a1
a2
b1
b2
c1
c2
d1
d2
Blastomere

1-5). The graph shows the mean time the embryos spent between divisions (no blastomeres dividing), in the 4-, 8-, and 16-cell stages. The sample sizes were 30 for the 4-cell stage, 32 for the 8-cell stage, and 15 for the 16-cell stage. Values are shown ± S.E.M.

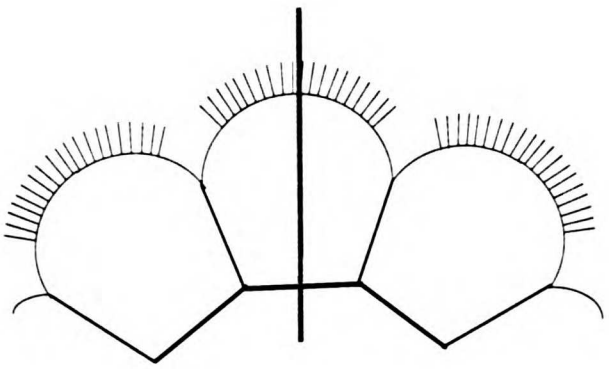

600
500
400
300
200
100
0
Time (min)
4-cell stage
8-cell stage
16-cell stage

1-6). The variability in the timing of compaction is illustrated by light micrographs made from a continous time-lapse film record of embryonic development. **a**. An embryo is completely "compacted" at the 4-cell stage. **b**. The same embryo is shown at the early blastocyst stage, as it cavitates (the arrow points to the nascent blastocoel). **c**. An embryo is shown that underwent no apparent cell flattening prior to fourth cleavage. **d**. The same embryo is shown at the blastocyst stage. In both **a** and **c** the frame pictured is just prior to any rounding up for subsequent cleavage, and thus represents the maximum amount of compaction seen in the given stage. Frames **e**, **g**, **i**, and **k** show four 8-cell embryos just prior to rounding up for fourth cleavage. Each of these embryos achieved a different degree of compaction during the 8-cell stage. Frames **f**, **h**, **j**, and **l** show these same four embryos at the late 16-cell stage, just prior to the start of fifth cleavage. The degree of compaction seen at this stage is similar for all four of these embryos. Scale bar = 50 μm.

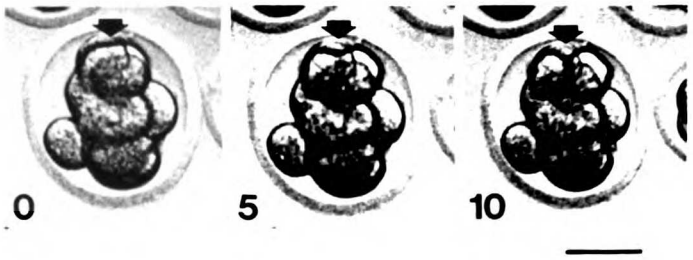

a
b
c
d
e
f
g
h
i
j
k
l

1-7). Schematic representations of anticlinal (A) and periclinal (B) division planes. Below each drawing is a time lapse series of an 8-cell blastomere undergoing that type of division, taken from cinemicrographic records. Numbers refer to time elapsed in minutes from the start of division of the blastomere. Arrows point to the blastomere of interest in each case. Scale bars = 50 μm.

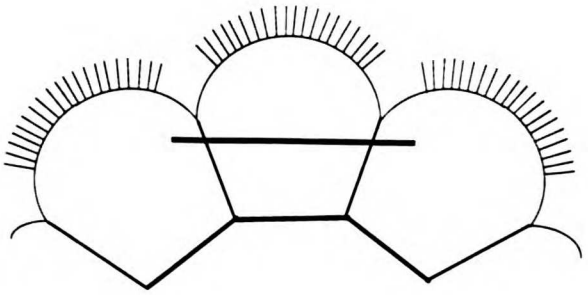
A



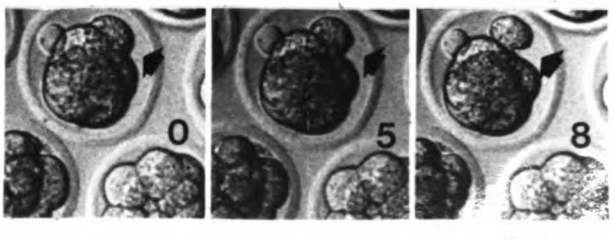
ANTICLINAL DIVISION



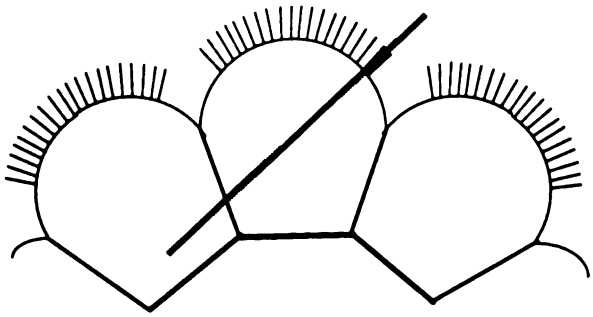
B



PERICLINAL DIVISION

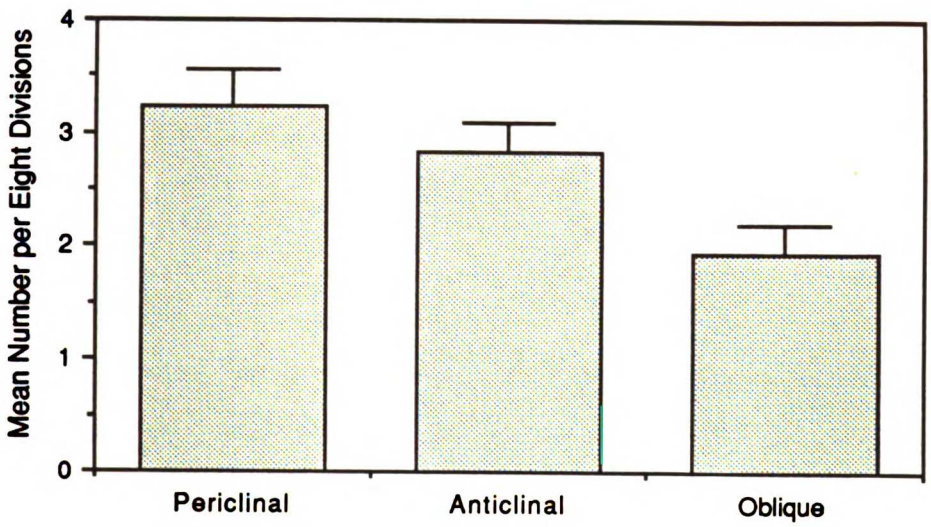


1-8). Schematic representation of the oblique division plane. Below the drawing is a time-lapse series of an 8-cell blastomere undergoing an oblique division, taken from a cinemicrographic record. Numbers refer to time elapsed in minutes from the start of division of the blastomere. Arrows point to the blastomere of interest. Scale bar = 50 μm.

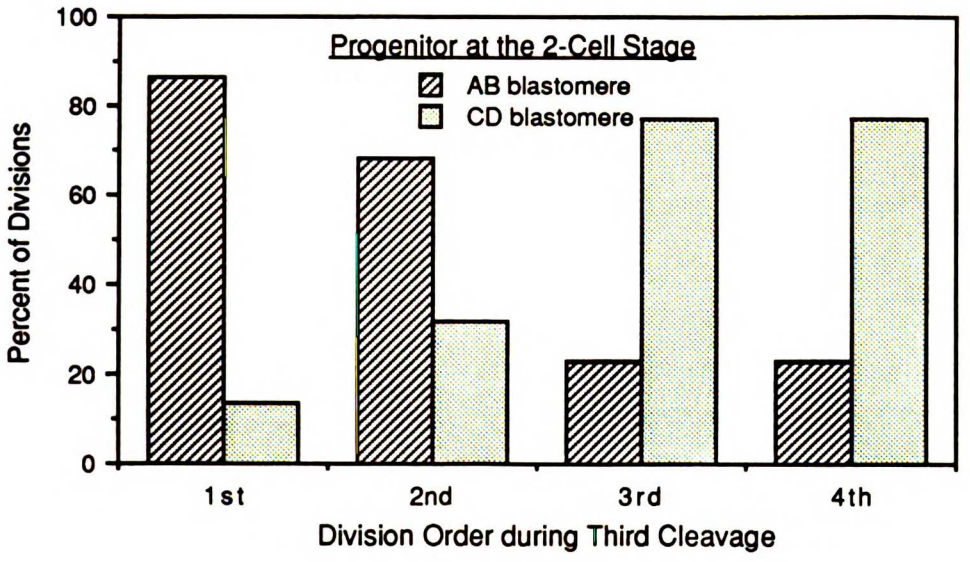


OBLIQUE DIVISION

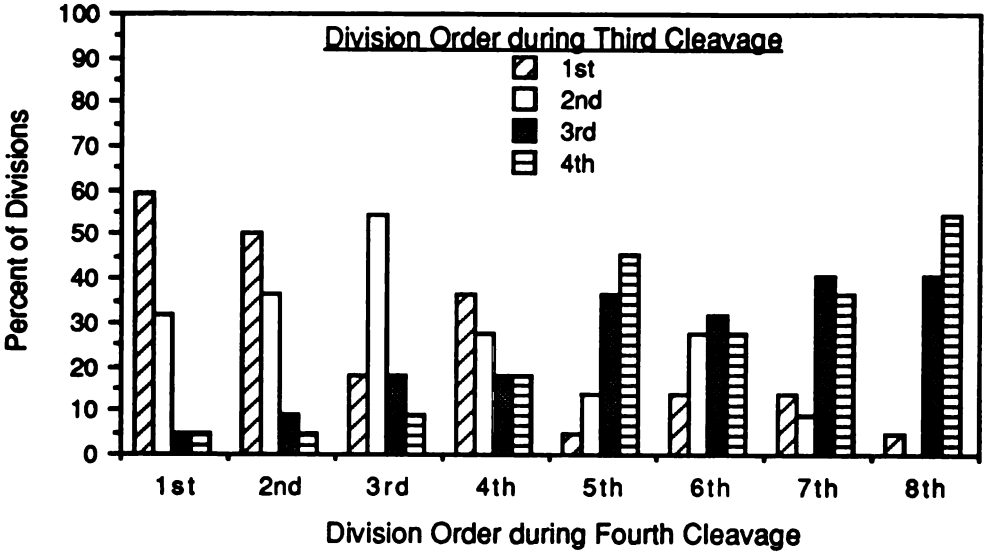
1-9). The graph shows the mean number of each type of division (anticlinal, periclinal, and oblique) per the eight divisions that comprise fourth cleavage. Values are shown ± S.E.M.


Mean Number per Eight Divisions
4
3
2
1
0
Periclinal
Anticlinal
Oblique

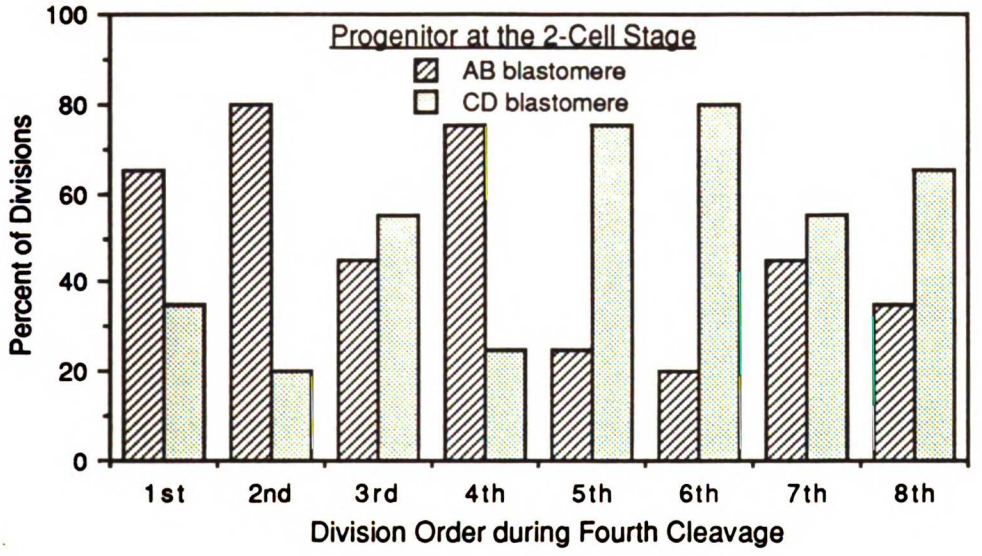
1-10). The graph shows the correlation between division order during second cleavage and division order during third cleavage. The AB blastomere is the earlier-dividing 2-cell blastomere during second cleavage, and the CD blastomere is the later-dividing 2-cell blastomere during second cleavage.


Progenitor at the 2-Cell Stage
AB blastomere
CD blastomere
Percent of Divisions
100
80
60
40
20
0
1st
2nd
3rd
4th
Division Order during Third Cleavage

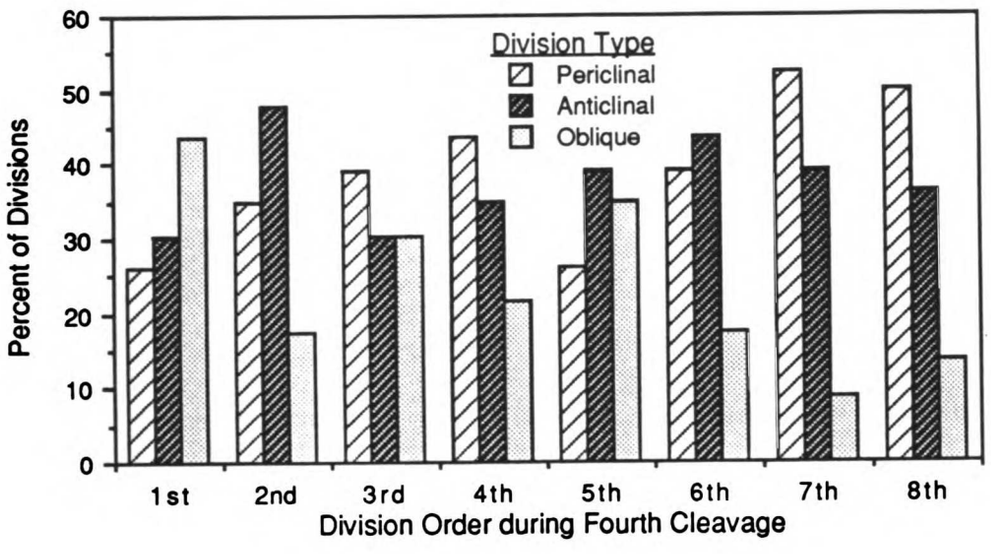
1-11). The graph shows the correlation between division order during third cleavage and division order during fourth cleavage.


Division Order during Third Cleavage
1st
2nd
3rd
4th
Percent of Divisions
100
90
80
70
60
50
40
30
20
10
0
1st
2nd
3rd
4th
5th
6th
7th
8th
Division Order during Fourth Cleavage

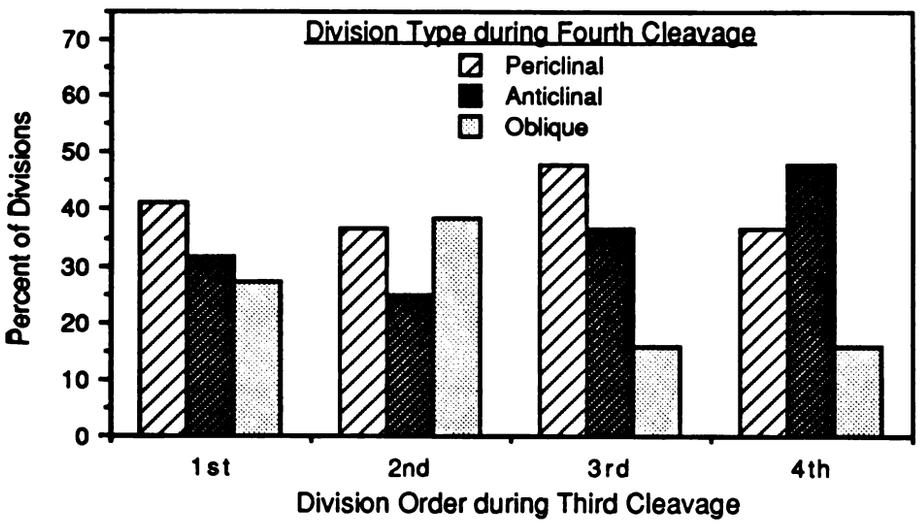
1-12). The graph shows the correlation between division order during second cleavage and division order during fourth cleavage. The AB blastomere is the earlier-dividing 2-cell blastomere during second cleavage, and the CD blastomere is the later-dividing 2-cell blastomere during second cleavage.


Progenitor at the 2-Cell Stage
AB blastomere
CD blastomere
Percent of Divisions
100
80
60
40
20
0
1st
2nd
3rd
4th
5th
6th
7th
8th
Division Order during Fourth Cleavage

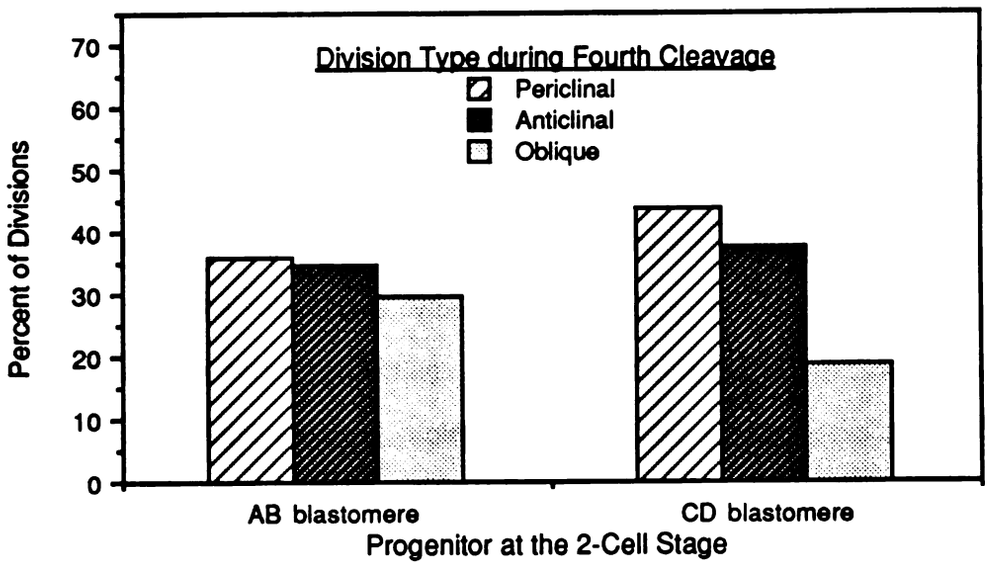
1-13). The graph shows the frequencies of the three types of divisions (periclinal, anticlinal, and oblique) for each position in the fourth cleavage division order.


Division Type
Periclinal
Anticlinal
Oblique
Percent of Divisions
60
50
40
30
20
10
0
1st
2nd
3rd
4th
5th
6th
7th
8th
Division Order during Fourth Cleavage

1-14). The graph illustrates the frequencies of the three types of divisions (periclinal, anticlinal, and oblique) for the 8-cell daughters of 4-cell blastomeres for each of the positions in the third cleavage division order.


Division Type during Fourth Cleavage
Periclinal
Anticlinal
Oblique
Percent of Divisions
70
60
50
40
30
20
10
0
1st
2nd
3rd
4th
Division Order during Third Cleavage

1-15). The graph illustrates the frequencies of the three types of divisions (periclinal, anticlinal, and oblique) for the 8-cell daughters of either the AB blastomere (earlier-dividing 2-cell blastomere) or the CD blastomere (later-dividing 2-cell blastomere).


Division Type during Fourth Cleavage
Periclinal
Anticlinal
Oblique
Percent of Divisions
70
60
50
40
30
20
10
0
AB blastomere
CD blastomere
Progenitor at the 2-Cell Stage

Chapter Two

Summary

The first morphogenetic event in preimplantation development of the mouse is the flattening of the eight-cell blastomeres upon one another in compaction. Accompanying compaction is the localization of microvilli on the apex of each blastomere, a process which has been termed polarization (Handyside, 1980; Johnson et al., 1981; Reeve, 1981; Reeve and Ziomek, 1981). These morphogenetic events are have been shown to involve microfilaments and perhaps microtubules (Ducibella and Anderson, 1975; Surani et al., 1980; Pratt et al., 1982; Maro and Pickering, 1984; Johnson and Maro, 1985; reviewed in Johnson and Maro, 1986). In order to further examine the role of the cytoskeleton in compaction and polarization, embryos were incubated in solutions of drugs that disrupt the cytoskeleton: cytochalasin B (a microfilament disruptive agent), colcemid (a microtubule disruptive agent), or lumicolchicine (an isomer of colchicine lacking activity against microtubules). Embryos were also incubated in medium containing tunicamycin, an inhibitor of N-linked glycosylation of glycoproteins, to determine the role glycosylated cell-surface proteins might have in compaction and polarization. Scanning electron microscope analysis of the effects of these agents on the early mouse embryo has yielded insights into the process of compaction. Cytochalasin B reversibly blocks or reverses cell flattening, and causes the formation of randomly localized clumps of irregularly-sized microvilli on the surface of the blastomeres, with some localization to the apex or lateral ring region. Colcemid treatment will accelerate the process of compaction somewhat but otherwise does not affect either cell flattening or polarization of surface microvilli, unless preceded by Pronase treatment of the embryos. Tunicamycin treatment does not affect cell flattening or polarization at the 8-cell stage but does cause gaps in intercellular adhesion. The evidence is consistent with a three-step compaction process involving (1) cell surface recognition and attachment of

lateral microvilli to adjacent blastomeres, (2) subsequent cell flattening with stabilization of the lateral ring of attaching microvilli, and (3) maintenance of the compacted and polarized state.

Materials and Methods

Embryo Culture

Female ICR mice (12 weeks old, Harlin) were superovulated by injection with 5 IU of pregnant mare's serum gonadotropin (Teikoku Hormone Mfg. Co., Japan) followed after 48 hours by an injection of 2.5 IU of human chorionic gonadotropin (hCG, Sigma). Following the second injection, the females were caged overnight with ICR males, and inspected the next morning for the presence of a vaginal plug, indicating insemination. Embryos were collected at the 2-cell stage (48 hours post-hCG) and cultured to the desired stage in microdrops (15-20 µl) of modified embryo culture medium (Biggers et al., 1971; Spindle, 1980). Incubation of culture drops is under a layer of mineral oil, at 37° C, in a humidified atmosphere of 5% CO2 in air. Zonae pellucida were removed enzymatically by brief incubation in Pronase (220 U/ml, Calbiochem) (Mintz, 1962). Following removal of the zona pellucida, embryos were allowed to recover in culture medium for 2-5 hours prior to experimental use.

Culture in Cytoskeletal Inhibitors

Four different reagents diluted in embryo culture medium were used: 1.) 0.5 µg/ml tunicamycin (Calbiochem, stock solution; 1 mg/ml in DMSO), 2.) 5µg/ml cytochalasin B (Sigma, stock solution; 0.5 mg/ml in DMSO), 3.) 5µg/ml colcemid (GIBCO, stock solution; 1 mg/ml in PBS), and 4.) 5 µg/ml beta- and gamma-lumicolchicines (Sigma, stock solutions; 1 mg/ml in DMSO). Controls included incubation in medium alone or appropriate concentrations of diluents (DMSO, PBS). No differences were seen between the different control embryos.

Previous dose-response data from our lab (Johnson and Calarco, 1980) and others (Surani, 1979) established 0.5 µg/ml tunicamycin as an effective

concentration for reversal of compaction. Published values for the concentrations of cytochalasin B used on embryos range from 5 to 10 μg/ml (Ducibella and Anderson, 1975; Surani et al., 1980). The former concentration was chosen, as it produced distinct effects on compaction in preliminary experiments. The effects of cytochalasin D (1μg/ml) were also examined, since this drug had been used in other studies (Surani et al., 1980; Pratt et al., 1982, Maro and Pickering, 1984). I found no significant differences between the effects of the two different cytochalasins on embryos.

Initial studies using 5μg/ml colcemid (a midrange value for published colcemid studies on embryos) proved effective in reversing compaction and was used in subsequent experimental studies. Lumicolchicine, an analog of colchicine that does not bind to tubulin or disrupt microtubules (Wilson et al., 1974) but does have the same side effects as colchicine (Mizel and Wilson 1972; Stadler and Franke, 1974) was used at 5μg/ml as a control for secondary toxicity.

Most experiments with cytochalasin B and colcemid utilized newly compacted 8-cell embryos selected after culture from the 2-cell stage (20-24 hours), but occasionally included some embryos undergoing the next round of division. Certain experiments with colcemid utilized uncompacted 8-cell embryos also selected after culture from the 2-cell stage. Tunicamycin and lumicolchicine experiments utilized embryos at the 2-cell stage. Culture was for varying time periods and experiments were repeated from two to five times. Two different methods of incubation were used: 1) embryos were cultured with zona pellucidae intact, treated with 0.5% Pronase for 5 minutes at 37° C (Mintz, 1962) after the appropriate culture period to remove the zona pellucida, and fixed, or 2) embryos were treated with Pronase prior to experimental incubations, cultured in individual microdrops, and fixed after

appropriate culture periods. Previous work has shown that treatment with Pronase does not induce changes in SEM morphology until the late blastocyst stage, when it causes rounding of the trophectoderm cells (Calarco, 1975). The ability of embryos to recover from the various experimental treatments was assessed by a 20-minute incubation in embryo culture medium prior to fixation. This recovery period was chosen empirically and was short enough to allow us to observe some of the initial events in recompaction.

Scanning Electron Microscopy

Embryos were fixed in 3% glutaraldehyde in PBS for two hours, and placed into empty ant pupae cases (Cooper and Ris, 1943; Mintz, 1971) which were then tied shut with a hair. This step facilitated later manipulations. Embryos were then post-fixed in osmium tetroxide (2% in PBS), dehydrated through a graded series of ethanols (25-100%) and critical-point dried out of liquid CO_2 in a Bomar SPC-900/EX critical point drier. Embryos were removed from the ant pupae case, mounted on double-stick tape, coated with 200 Å of gold, and observed and photographed on a Cambridge S-150 scanning electron microscope.

Cinemicrography

Embryos were placed in a pre-equilibrated microdrop of culture medium containing 5 µg/ml colcemid under oil in a Falcon 35 mm tissue culture dish . The cover of the dish was inverted and two holes bored into opposite sides; one a pinhole, the other large enough to accept a syringe tip. The cover was then sealed onto the dish using high vacuum grease (Dow Corning). A piece of tubing was inserted into the large hole in the cover, and attached at at the other end to a tank containing 5% CO2 in air. The flow of gas was adjusted to a level that did not disturb the surface of the oil.

The chamber was then placed into a stage heater (Steier, 1975) controlled by a proportional temperature controller (Yellow Springs, model 72) on the stage of a Nikon Diaphot inverted microscope outfitted with Hoffman Modulation Contrast optics. The embryos were filmed over a period of 12 hours using a 10X objective, and their development recorded on Kodak Tri-X reversal film with a Bolex H-16 camera controlled by a Nikon CFX intervalometer. Frames were taken at 30-second intervals, and the film analyzed on a NAC model 16C analysis projector.

Results

A. Cytochalasin

Compacted 8-cell embryos incubated in cytochalasin B (CB) were completely decompacted after 1.5 hours. No changes were seen by light microscopy after longer incubations (4 or 24 hours) and no cell division occurred during the treatment period. CB effects were readily reversible; CB-treated embryos rinsed and allowed to recover in normal medium for 20 minutes rapidly recompacted. Whether embryos were treated with Pronase to remove their zonae pellucidae prior to or subsequent to their incubation in CB had no effect on the severity of CB effects or the time course of decompaction. Preliminary experiments with cytochalasin D showed no significant differences from treatment with CB.

Scanning electron microscopy (SEM) examination of embryos after 1.5 hours incubation showed the blastomeres to be rounded and separate from one another, with occasional bleb-like protrusions and fewer microvilli than control embryos (Figure 2-1). Most of the microvilli were in irregularly sized clumps randomly distributed over the surface of the blastomeres (Figure 2-1b, c). The microvilli within these clumps were of many different lengths, with some many times longer than others (see also below). When embryos were allowed to recover in culture medium for 20 minutes, the blastomeres began to flatten upon one another, and the surface microvilli became more regular in size and distribution (Figure 2-1d). Apical polarization of microvilli was usually seen in these embryos (Figure 2-1d).

Embryos examined after 4 hours in CB were completely decompacted and had even fewer microvilli than 1.5 hour CB embryos (Figure 2-2a, b). Remaining microvilli were again of variable length, but were arrayed in larger and fewer clumps

than in the earlier sample (Figure 2-2c, d). Localization of microvilli to the apical region was seen in some embryos, although more often the clumps of microvilli were randomly distributed on the cell surface. Occasionally, microvilli were seen to be organized into a dense ring laterally encircling the blastomere (Figure 2-2a).

After 24 hours in CB, microvilli were further reduced in number and were arranged in clumps, as described for earlier samples. These clumps were randomly arranged on the blastomere surface; no specific apical or lateral localizations were seen (Figure 2-3a). After only 20 minutes recovery in embryo culture medium several striking changes were evident, including a more even distribution of short microvilli, and prominent filiform protrusions stretching between adjacent cells at their basolateral margins (Figure 2-3b). Control embryos were morulae and blastocysts at this time.

B. Colcemid

Uncompacted 8-cell embryos placed in colcemid (CM) proceeded to compact normally during the first 10 hours of culture as seen by LM observation, cinemicrographic analysis, and SEM (Figures 2-4 and 2-5). The rate at which these embryos flattened was accelerated somewhat over that seen in controls (Figure 2-4). These embryos and those placed in CM after compaction subsequently decompacted at approximately the same time control embryos were beginning fourth cleavage. CM-treated embryos did not divide further, and remained decompacted until cultures were terminated at 24 or 48 hours. Embryos rinsed and allowed to recover in normal medium did not recompact, although some increase in cell flattening was seen. Uncompacted 8-cell embryos treated with Pronase to remove the zona pellucida prior to incubation in CM did not compact in up to 10 hours of culture. Two-cell embryos cultured in lumicolchicine (LC), an analogue of colchicine that does not bind to tubulin,

underwent cleavage, compaction, and blastocoel formation at the same rate as controls and were morphologically normal by SEM examination. Pronase treatment at the 8-cell stage followed by incubation in LC had no effect on subsequent compaction.

Preselected uncompacted and compacted 8-cell embryos were incubated separately in CM and observed by SEM as described below:

1). Uncompacted embryos placed in colcemid. Uncompacted embryos were incubated in colcemid-containing medium for four or ten hours, then fixed and examined by SEM. Before the embryos compacted,.the blastomeres were rounded and regularly shaped, although a small amount of blebbing was seen (Figure 2-5a, b). These embryos were similar to controls. Polarization of microvilli to the apical region of the blastomeres was not seen in embryos where cell flattening had not begun (Figure 2-5a, b). After compaction, CM-treated embryos were similar to controls morphologically and had a characteristic polarized appearance, i.e. apical localization of microvilli and areas of smooth membrane in the regions of cell-cell contact (Figure 2-5c, d).

2). Compacted embryos placed in CM. Compacted embryos were incubated in CM-containing medium for four or ten hours, then fixed for SEM examination. Embryos were partially decompacted after 4 hours in CM (Figure 2-6a, b). The cell-surface microvilli were evenly distributed over the free surface of the blastomere rather than in a polarized configuration, and usually appeared shorter and fewer in number than in control embryos (Figure 2-6a, b). After 10 hours in CM, embryos were completely decompacted (Figure 2-6c, d). After 20 minutes recovery in culture medium many lateral microvilli were observed extending from one blastomere to another, but organization of microvilli into an apical pole was not observed (Figure 2-6c, d). In fact, the apices of the blastomeres often appeared to have fewer microvilli

than the lateral regions (Figure 2-6c, d). Some blastomeres appeared irregularly shaped.

Both embryos placed in CM-containing medium before compaction and those placed in CM-containing medium after compaction were morphologically similar after a 24 hour incubation. All were decompacted and none showed apical polarization of microvilli (Figure 2-7a, b). Upon 20 minutes recovery in culture medium, embryos showed increased intercellular apposition and numerous long microvilli attached to adjacent blastomeres at their lateral surfaces (Figure 2-7c). These attaching microvilli usually appeared rigid and straight but in many embryos were limp and sinuous (Figure 2-7d, e). Control embryos had reached the late morula or early blastocyst stage after 24 hours.

C. Colcemid and Cytochalasin

Compacted 8-cell embryos incubated for 24 hours in combination of CM and CB (5 μg/ml each) were similar in appearance to embryos cultured in CB alone (Figure 2-8). The blastomeres were rounded and completely decompacted, with few microvilli. The characteristic apical polarization pattern observed in controls was not seen; microvilli were clumped together in randomly localized patches (Figure 2-8a, b). When embryos were allowed to recover in normal culture medium for 20 minutes following a 24 hour incubation in CM+CB, striking changes were seen. All embryos showed increased numbers of MV located in the basal-lateral areas of contact between blastomeres. These MV were evenly distributed rather than clumped, although clumps of microvilli were still seen on the apical portion of the blastomeres (Figure 2-8c). Increased cell-cell interactions were observed via these MV, which appeared to connect each blastomere to its neighbors, although the blastomeres remained rounded.

D. Tunicamycin

Two-cell embryos cultured in tunicamycin (TM) appeared by light microscopy to divide and compact at the same rate as control embryos in the first 24 hours of culture. During longer incubations, embryos showed increasing numbers of rounded, decompacted blastomeres, and by 48 hours in culture were fully decompacted. Embryos cultured in tunicamycin did not form blastocysts.

Control embryos examined by SEM after 24 hours in culture ranged from the 8- to the 16-cell stage. Many had a polarized appearance marked by apical microvilli and smooth membrane along regions of cell contact (Figure 2-9a, b, c). Cell number was similar to controls in embryos cultured 24 hours in TM. In general, embryos with fewer cells were the most compacted. Discontinuities in blastomere apposition were seen in all embryos after TM treatment but were not seen in control embryos at the same stage. These discontinuities were evident even in those embryos that appeared completely compacted by LM, and were usually observed in areas where three blastomeres were in contact (Figure 2-9a, b, c). With increasing amounts of time in TM, embryos appeared to have progressively fewer microvilli than controls and these were usually localized in the apical region of the cell (Figure 2-9a, b, c). Embryos with from 9 to 16 cells had fewer microvilli than 8-cell embryos. Intercellular connections often took the form of broad loboform protrusions ending in short filiform attachments on the adjoining blastomeres (Figure 2-9c).

Embryos fixed after 48 hours in TM ranged from 12 to 20 cells. Although they appeared decompacted by LM observation, a considerable amount of intercellular apposition was still present (Figure 2-9d, e). Intercellular contacts were maintained via filiform or loboform protrusions as described above (Figure 2-9d, e). A large proportion of embryos was nearly devoid of MV after 48 hours in TM, with only a few

remaining laterally in areas of intercellular contact (Figure 2-9d, e). These embryos appeared to have more blastomeres than those with more MV, suggesting that the effects of TM treatment become more pronounced as development proceeds. Control embryos were morulae and early blastocysts after 48 hours, with 16-32 cells.

Discussion

The results of this study confirm and extend earlier observations that the cytoskeleton (Ducibella and Anderson, 1975; Surani et al., 1980; Pratt et al., 1981, 1982; Maro and Pickering, 1984; Johnson and Maro, 1985) and cell-surface glycoproteins (Surani, 1979; Atienza-Samols et al., 1980; Surani et al., 1981; Pratt et al., 1982) are involved in compaction of the 8-cell mouse embryo. In the present study, the use of SEM has provided new information and a more complete description of the morphological effects of disruption of these cellular components, as well as the initial events of recovery from treatment. From these studies I have gained several insights into the role of the cytoskeleton in compaction, and a clearer understanding of compaction itself.

Compaction, as defined in the present study, includes two interrelated processes; cell flattening and cell surface polarization. In my studies, I used intact embryos rather than dissociated blastomeres, and so my criterion for polarization of the blastomeres was the presence of a clearly defined pole of microvilli located on the apex of the blastomere, surrounded by an area of smooth membrane that extended to the area of cell-cell contact between the blastomeres. Other reports in which these types of experiments have been done have involved dissociating the embryo into component blastomeres prior to examining them for the effects of the experimental treatment, in order to better gauge the state of polarization of the blastomeres (Pratt et al., 1982; Maro and Pickering, 1984; Johnson and Maro, 1985, 1986). The reason this was not done in the present study is that the event termed "polarization" was originally defined as the formation of a discrete apical pole of microvilli surrounded by an area of smooth membrane, which occurred at the 8-cell stage during compaction (Handyside, 1980; Reeve and Ziomek, 1981). In normal embryos viewed by SEM, this

discrete pole of microvilli often disappears by the 16-cell stage, leaving the free surface of the blastomere evenly covered with microvilli (Calarco, 1975). The blastomeres of these embryos are clearly part of a polarized epithelium, and as such have microvilli only on their free surfaces. However, it is not clear that this polarity and the event termed "polarization" at the 8-cell stage are exactly equivalent. The mouse embryo also displays a polarized localization of various cytoplasmic and cell surface entities such as myosin, spectrin, and alkaline phosphatase at the 2- and 4-cell stages, and thus could be considered to be polarized throughout the cleavage period (Izquierdo et al., 1980; Sobel, 1983; Sobel and Alliegro, 1985; Handyside et al., 1987). In doing the above-described experiments I was concerned specifically with the compaction-related polarization of microvilli, therefore, I used only the strictest definition of the term, as described above, in interpreting my results. Because of this, some confusion may arise in comparing the results of other studies to those reported here; this was taken into account in the following discussion.

Cytochalasin B

The cytochalasin B experiments clearly demonstrate that cell flattening is microfilament dependent, in agreement with earlier studies (Ducibella and Anderson, 1975; Surani et al., 1980; Pratt et al., 1981, 1982). I found that compacted embryos placed in CB decompact within 1.5 hours, and remain uncompacted as long as they remain in CB. The effect is readily reversible, since embryos allowed to recover in normal culture medium will begin to compact within 20 minutes.

In contrast to its effect on cell flattening, CB does not completely abolish polarization during short incubations. However, significant effects on the organization of the microvilli are seen after 1.5 hours in CB, which become more pronounced with increased time of culture in the drug. Generally, the microvilli are arranged in many

smaller clumps, which are randomly located on the free surface of the blastomere. After short incubation periods these clumps are small and numerous, after longer periods they are somewhat larger and much less numerous; the overall number of microvilli becomes much smaller with increasing time in culture. Similar patterns have been found by others (Pratt et al., 1982; Johnson and Maro, 1984, 1985) and interpreted as showing continued polarization of the blastomere. It is not clear that this is so; certainly the microvilli are not distributed homogeneously, but then neither are they polarized even in the sense of being restricted to one region of the blastomere as is seen in control embryos. Polarization in the latter sense is seen to some degree in embryos cultured for short amounts of time in CB, but it is not consistent, and decreases with increasing time in culture in CB. Figure 2-1 shows that clumps of microvilli can be found on all parts of the blastomere surface of CB treated embryos, including the basal and lateral regions that would normally be in contact with the adjoining blastomeres in the compacted embryo. In several cases the lateral microvilli were more numerous than those found in the apical region (Figure 2-2), perhaps because they were stabilized by interaction with adjoining blastomeres prior to CB treatment.

The ability of blastomeres to polarize is not lost after short incubations in CB (1.5 hours) despite the changes effected during the course of the treatment; after a 20 minute recovery period the blastomeres were again observed to have the characteristic apical pole of microvilli (Figure 2-1d). However, embryos allowed to recover after longer incubations do not have microvilli apically, but laterally, in the areas of intercellular contact (Figure 2-3b)

There appear to be two populations of microfilaments in the 8-cell blastomeres; those that are in a state of rapid turnover, and thus are susceptible to

depolymerization by CB (Lin et al., 1980), and those that are more stable and turn over at a much slower rate, making them less sensitive to CB treatment. My results suggest that cell flattening and polarization are mediated by microfilaments that are actively turning over, while the cell surface microvilli are comprised of those turning over more slowly. This latter population of microfilaments is not completely insensitive to CB however, since the surface microvilli become very irregular in size, and reduced in number as the length of time of CB treatment increases. The presence of populations of microfilaments with differing sensitivities to cytochalasin treatment has been demonstrated by others, both in mouse embryos (Pratt et al., 1981, 1982) and other cell types (Wessells et al., 1971; Goldman, 1972).

Colcemid

Treatment with colcemid did not affect compaction or polarization; uncompacted 8-cell embryos treated with CM compacted somewhat more rapidly than controls, and were indistinguishable morphologically. This is in agreement with the findings of others, using different microtubule disrupting agents such as colchicine or nocodazole (Ducibella, 1982; Maro and Pickering, 1984; Johnson and Maro, 1985), although some effect of nocodazole was seen on the extent of the pole of microvilli (Maro and Pickering, 1984). Embryos cultured in nocodazole were found to compact sooner than do controls, whereas those cultured in Taxol (an agent causing uncontrolled polymerization of tubulin) fail to compact (Maro and Pickering, 1984; Johnson and Maro, 1985). These results, taken together with ours, suggest that microtubules do not play an active role in cell flattening or polarization, but that microtubule reorganization or modification is part of the compaction process.

When embryos were incubated in CM for periods of 24-48 hours, I observed that blastomere flattening and polarization of microvilli were not maintained. This

probably represents the rounding of the blastomeres for the next mitosis,since the blastomeres of CM treated embryos rounded up at approximately the same time as those of control embryos were rounding up for fourth cleavage. The absence of polarization after these longer incubations may mean that microtubules are involved in stabilization of the polarized distribution of microvilli, or that the normal spreading of the apical pole during mitosis (Johnson and Ziomek, 1981) is continuing in the presence of CM and in the absence of cell division. The fact that there were often fewer microvilli in the apical region than in the lateral regions (Figures 2-6c, d; 2-7b) supports the former interpretation.

Tunicamycin

My results with tunicamycin (TM are in fundamental agreement with the LM observations of Surani et al. (1981). In addition, due to the greater resolution of the SEM, I have shown that TM inhibition of glycoprotein synthesis affects even the earliest stages of compaction. Discontinuities in blastomere apposition were seen in all embryos after TM treatment, even those that appeared completely compacted by LM. These discontinuities were not seen in control embryos at the same stage. In accordance with the findings of Surani et al. (1981), but in contrast to those of Pratt et al. (1982), I found that embryos incubated in TM developed normally to the 8-cell stage, as judged by timing of cleavage and initiation of compaction. The delay in compaction, and lower number of cleaving embryos found by Pratt et al. (1982) may be due to their use of a higher concentration of TM. During later stages embryos were progressively more affected by TM culture, as seen by the reduced numbers of microvilli, absence of polarity of microvilli, complete decompaction of embryos, and reduced numbers of blastomeres as compared to controls.

Decompaction of embryos in TM is probably due to lack of certain cell surface glycoproteins (Surani et al., 1981). I cannot exclude the possibility that my tunicamycin had some inhibitory effect on protein synthesis. However, Surani (1979) found that incorporation of leucine into proteins was essentially normal at TM concentrations ranging from 0.25 to 2 μg/ml. The concentration I used (0.5 μg/ml) is within this range. In addition, Grabel and Martin (1983) found no inhibition of leucine incorporation in teratocarcinoma cells at TM concentrations from 0.2 to 0.5 μg/ml. The two above studies and my study used TM from the same source. This led us to interpret the observed effects as due to inhibition of N-linked glycosylation rather than inhibition of protein synthesis. The present work shows that even when decompacted, TM-treated embryos are able to maintain some degree of intercellular adhesion and apposition. This may be due to incomplete inhibition of glycosylation by TM (Surani et al., 1981) or perhaps to other classes of cell surface molecules, e.g., glycolipids.

I found that incubation of 2-cell embryos in TM for 24 hours did not block polarization of microvilli at the 8-cell stage. Although reduced in number, microvilli were localized to the apex of most blastomeres; lateral microvilli were not prominent. However, at the 16-cell stage, (48 hours in TM) polarization was not observed; remaining microvilli were predominantly found along lateral contiguous surfaces. This is in contrast to Pratt et al. (1982) who found reduced numbers of polarized blastomeres in embryos at the 8-cell stage in TM. This may again be due to the higher concentration of TM used in that study.

The importance of cell surface moieties to compaction was also indicated by the fact that Pronase treatment of uncompacted embryos prior to colcemid incubation completely inhibited compaction. Pronase-treated 8-cell embryos cultured in

lumicolchicine did compact, which suggests that microtubules are involved in the replacement of cell-surface adhesion proteins removed during zona digestion.

Lateral microvilli appear to be important in cell flattening and compaction. I consistently observed the reappearance of microvilli in areas of cell-cell apposition upon recovery from CB or CM treatment, coincident with an increase in intercellular apposition. This was also apparent in experiments using a combination of CM and CB. An earlier study (Calarco and Epstein, 1973) shows that long lateral microvilli are present in normal, untreated embryos as well, suggesting that these lateral microvilli are an active part of the compaction process. It is also in this region that zonular tight junctions will eventually form (Calarco and Brown, 1969; Magnuson et al., 1977).

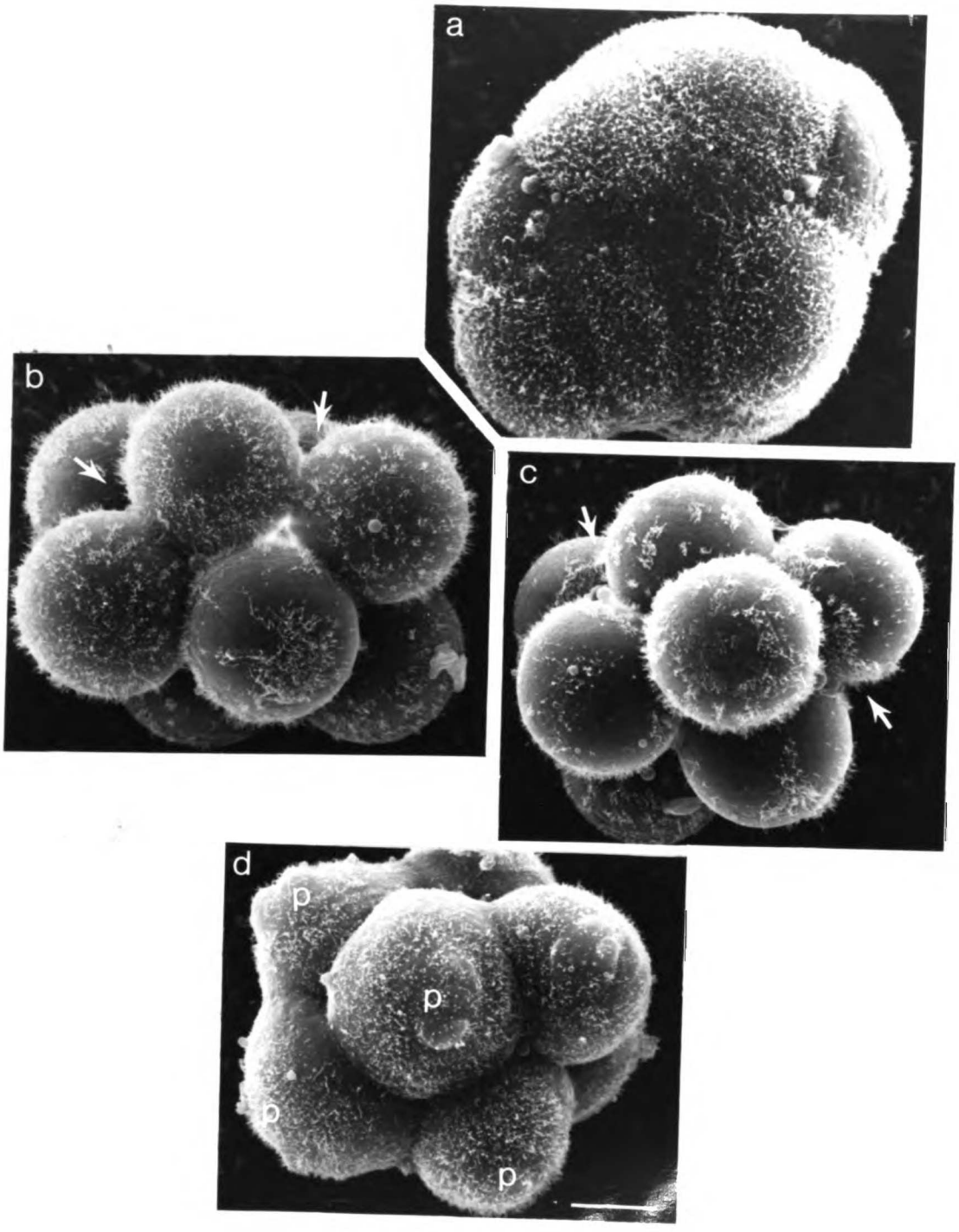
In summary, my experimental evidence and that of others is consistent with a three-part compaction process:

1.) The attachment of lateral microvilli to the cell surfaces of neighboring cells. I view this as a surface event likely to be mediated by the interaction of specific cell surface molecules. This can be inferred from experiments which directly affect the cell surface and block compaction, e.g., Pronase digestion coupled with colcemid incubation reported here, the TM block of N-linked protein glycosylation (Surani, 1979), and antibody treatment of living embryos (Kemler et al., 1977; Johnson et al., 1979; Damsky et al., 1983; Shirayoshi et al., 1983).

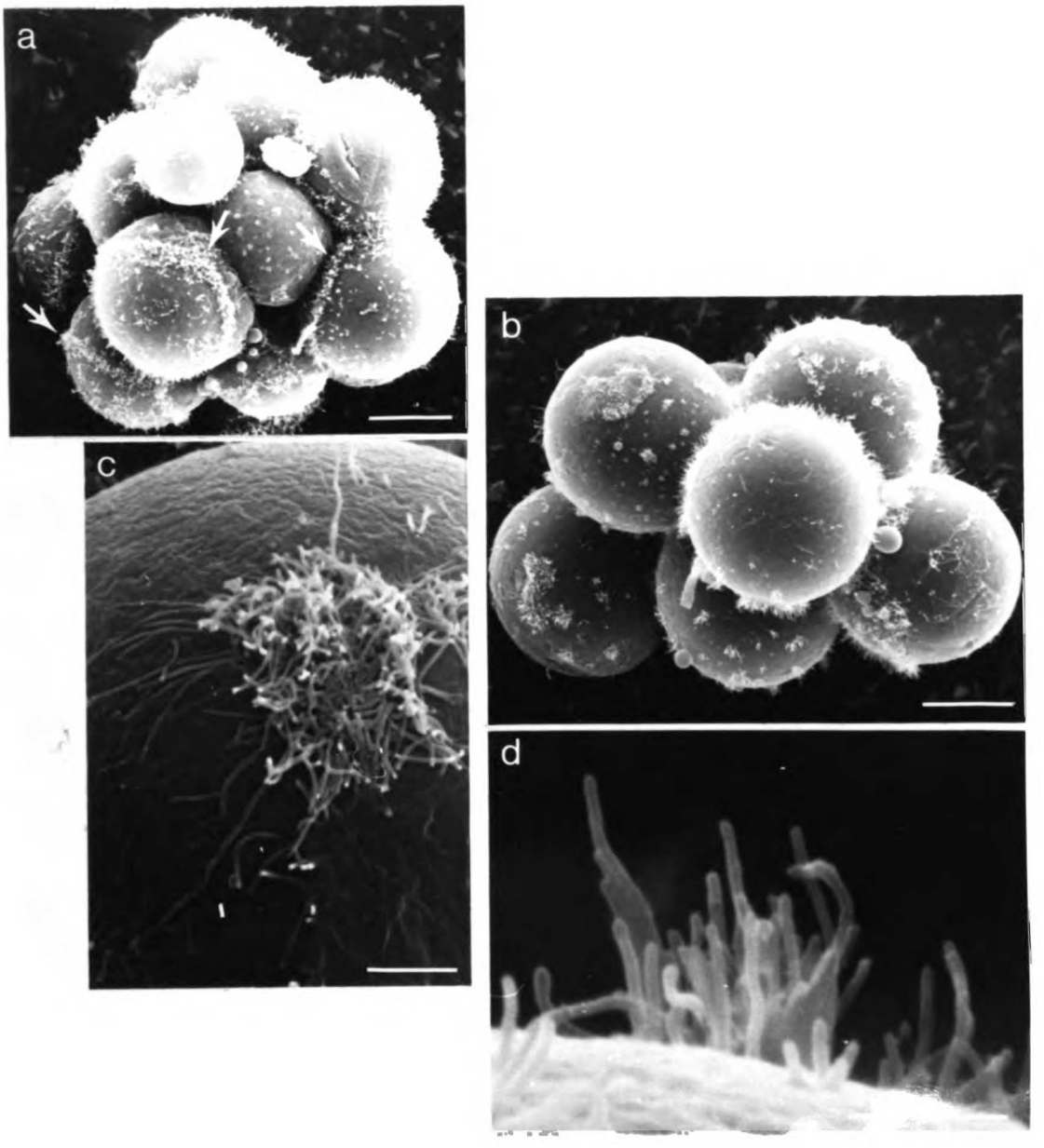
2.) The flattening of adjacent blastomeres against one another coincident with the stabilization of the lateral ring of attaching microvilli. This step is cytochalasin sensitive and, therefore, dependent on microfilaments. Consistently, my experiments showed the simultaneous reappearance of numerous microvilli in the area of the lateral ring upon recovery from cytochalasin or colcemid, and increased intercellular adhesion and apposition. Undoubtedly, increased membrane fluidity and cell deformability must also play a part in achieving close cell apposition (Pratt, 1978).

3.) The stabilization and maintenance of the compacted and polarized configuration. This is also cytochalasin-sensitive, and thus microfilament-dependent, although microtubules may be involved to some extent with the stabilization of polarization, because CM treatment for 10-24 hours resulted in the loss of surface polarity.

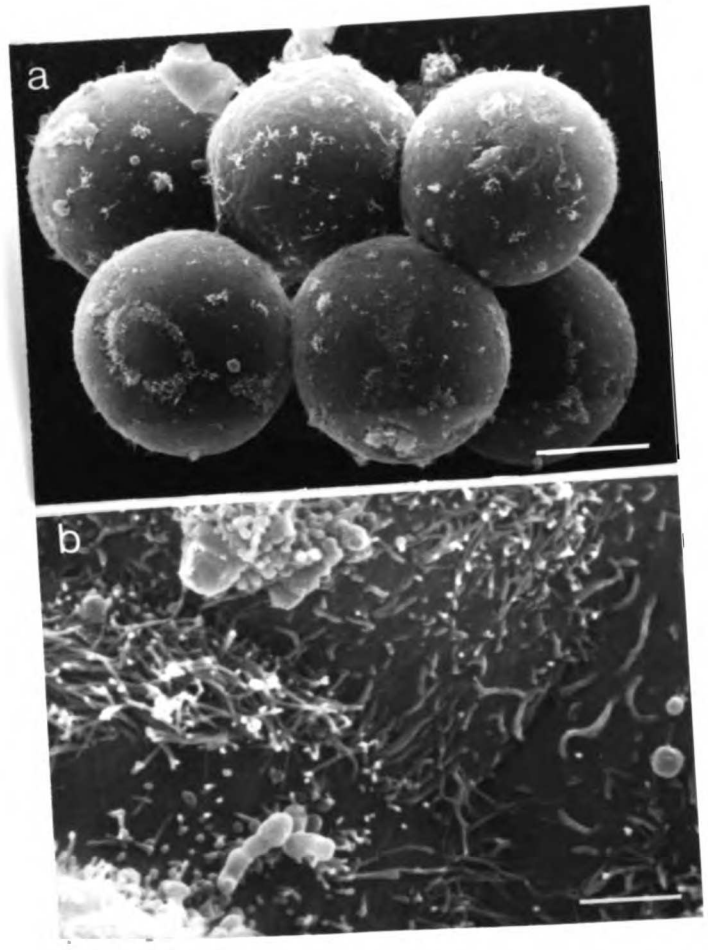
2-1). Scanning electron micrographs of mouse embryos treated for 1.5 hours with cytochalasin B. **a**. Control compacted embryo after 24 hours culture from the 2-cell stage. Note the close apposition of blastomeres to one another, and the polar appearance of some blastomeres, with an apical localization of microvilli and smooth membrane in the regions of cell contact. **b**. An 8-cell embryo cultured for 1.5 hours in 5 μg/ml cytochalasin B. The embryo is decompacted, and the microvilli are in small clumps on the surface of the blastomere. Arrows point to lateral areas of blastomeres containing microvilli. **c**. An 8-cell embryo cultured for 1.5 hours in 5 μg/ml cytochalasin B. Again, the arrows point to lateral localization of microvilli. **d**. An 8-cell embryo cultured 1.5 hours in cytochalasin B and allowed to recover for 20 minutes in embryo culture medium (compare with **b** and **c**). Microvilli are essentially normal in number and distribution, and polarization to the apical region is evident (P). Compaction of the blastomeres has also begun. Scale bar = 10 μm.


a
b
c
d
p
p
p
p

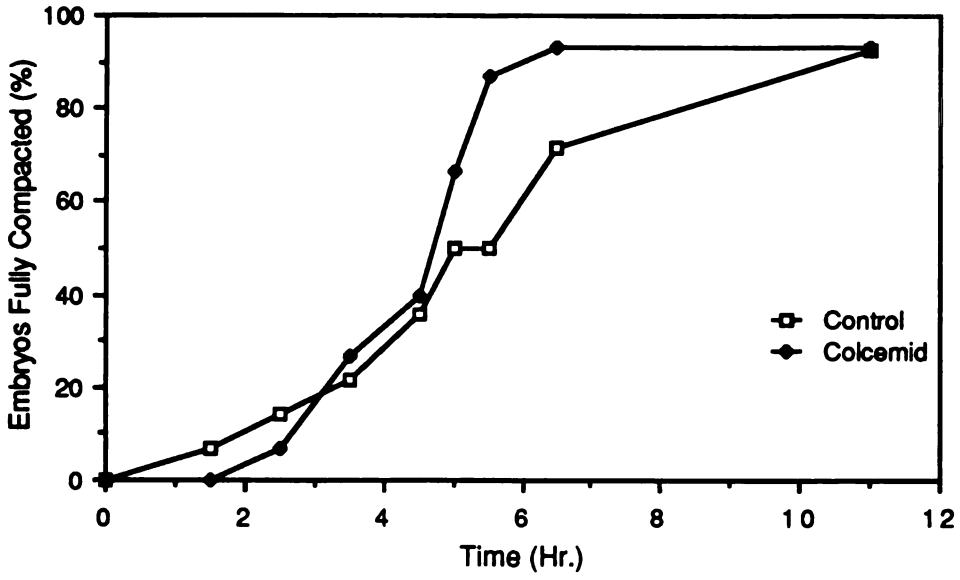
2-2). Scanning electron micrographs of embryos treated 4.0 hours with cytochalasin B. **a**. Arrows denote the dense lateral rings of microvilli encircling certain blastomeres. Microvilli are again localized in small clumps on the blastomere surface. This embryo is older, and has begun fourth cleavage. Scale bar = 10 μm. **b**. An 8-cell embryo after 4 hours in cytochalasin B. The clumps of microvilli are larger and fewer in number than in embryos in Figure 2-1. Again there is a lack of any consistent localization of these clumps on the surface of the blastomeres. Scale bar = 10 μm. **c**. A portion of an 8-cell embryo cultured for 4 hours in 5 μg/ml cytochalasin B, illustrating a large clump of microvilli and a large expanse of smooth membrane. Scale bar = 2μm. **d**. Clump of microvilli on the surface of one blastomere of an embryo treated 4 hours with cytochalasin B. Note the variability in length of the microvilli. Scale bar = 1 μm.


a
b
c
d

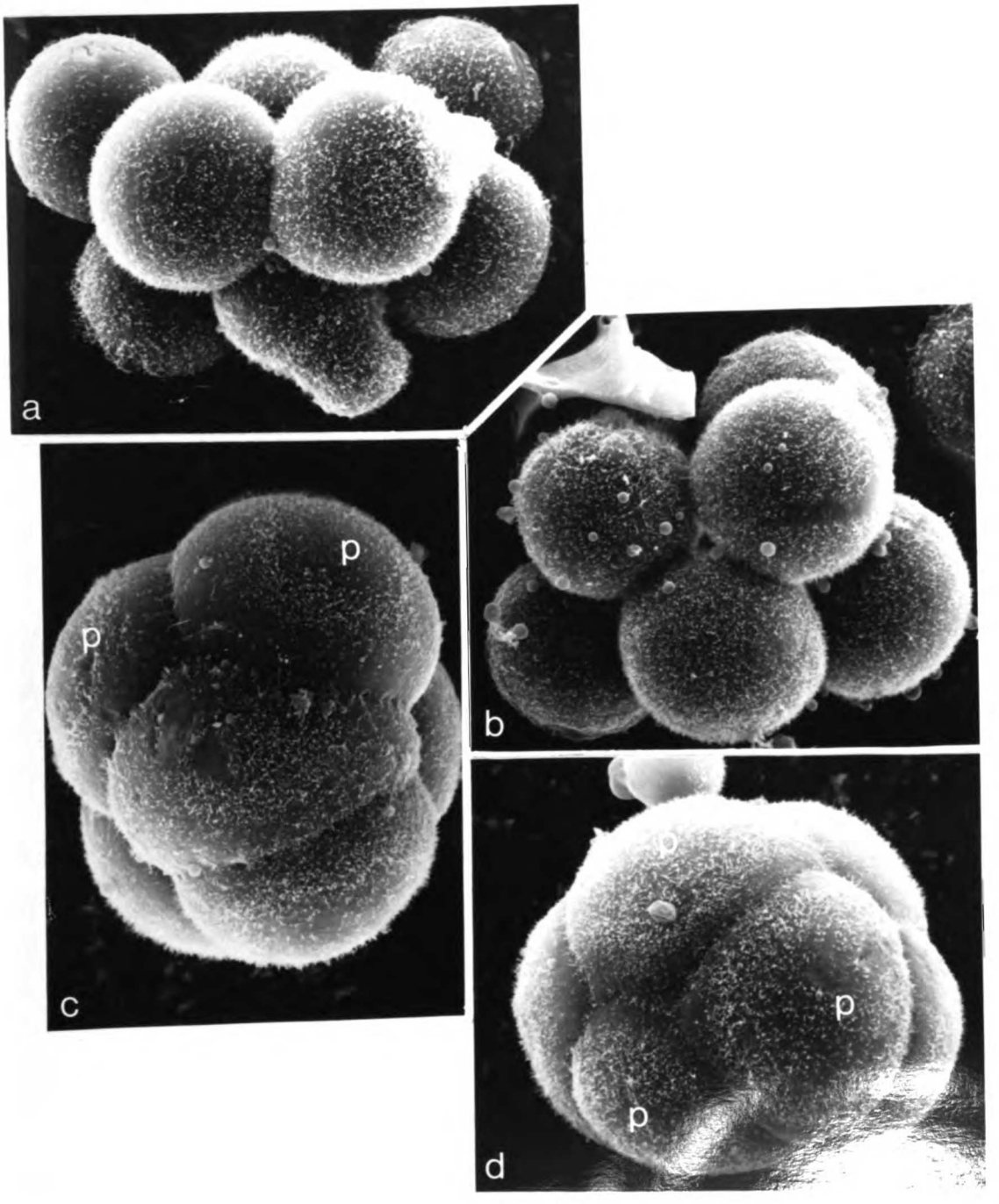
2-3). Scanning electron micrographs of embryos cultured 24 hours from the 8-cell stage in cytochalasin B. **a**. A portion of an 8-cell embryo, showing complete decompaction, fewer surface microvilli, and absence of any polarization of remaining microvilli. Scale bar = 10 μm. **b**. A portion of an 8-cell embryo cultured 24 hours in cytochalasin B and allowed to recover for 20 minutes. This micrograph shows the region of cell-cell contact between three blastomeres. Note the increased number of microvilli and their concentration in the area of apposition. Scale bar = 2 μm.


a
b

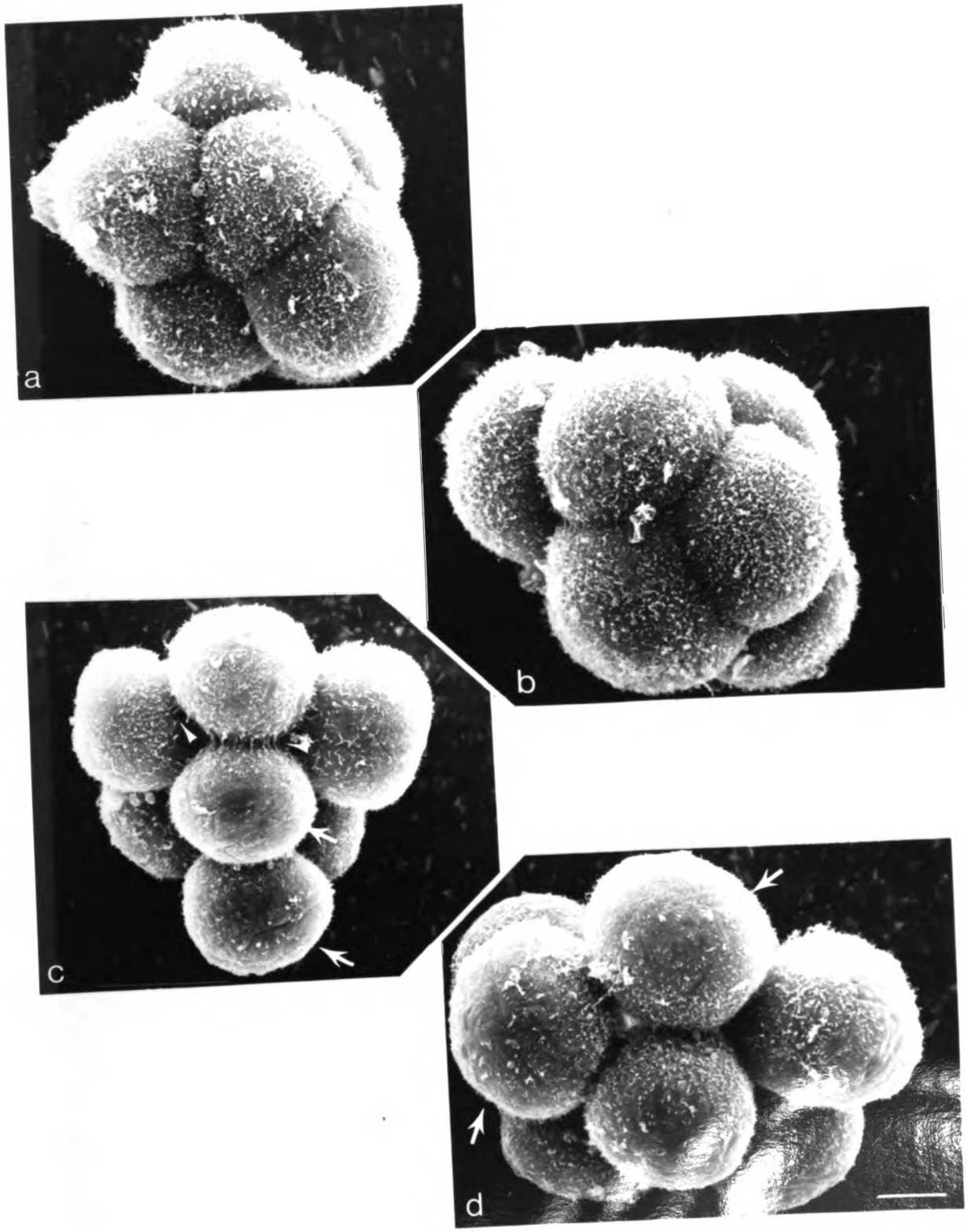
2-4). Graph showing the number of embryos becoming completely compacted with increasing amount of time in either control medium or medium containing 5 μg/ml colcemid. The number of embryos in each sample was 15.


100
80
60
40
20
0
Embryos Fully Compacted (%)
0
2
4
6
8
10
12
Time (Hr.)
Control
Colcemid

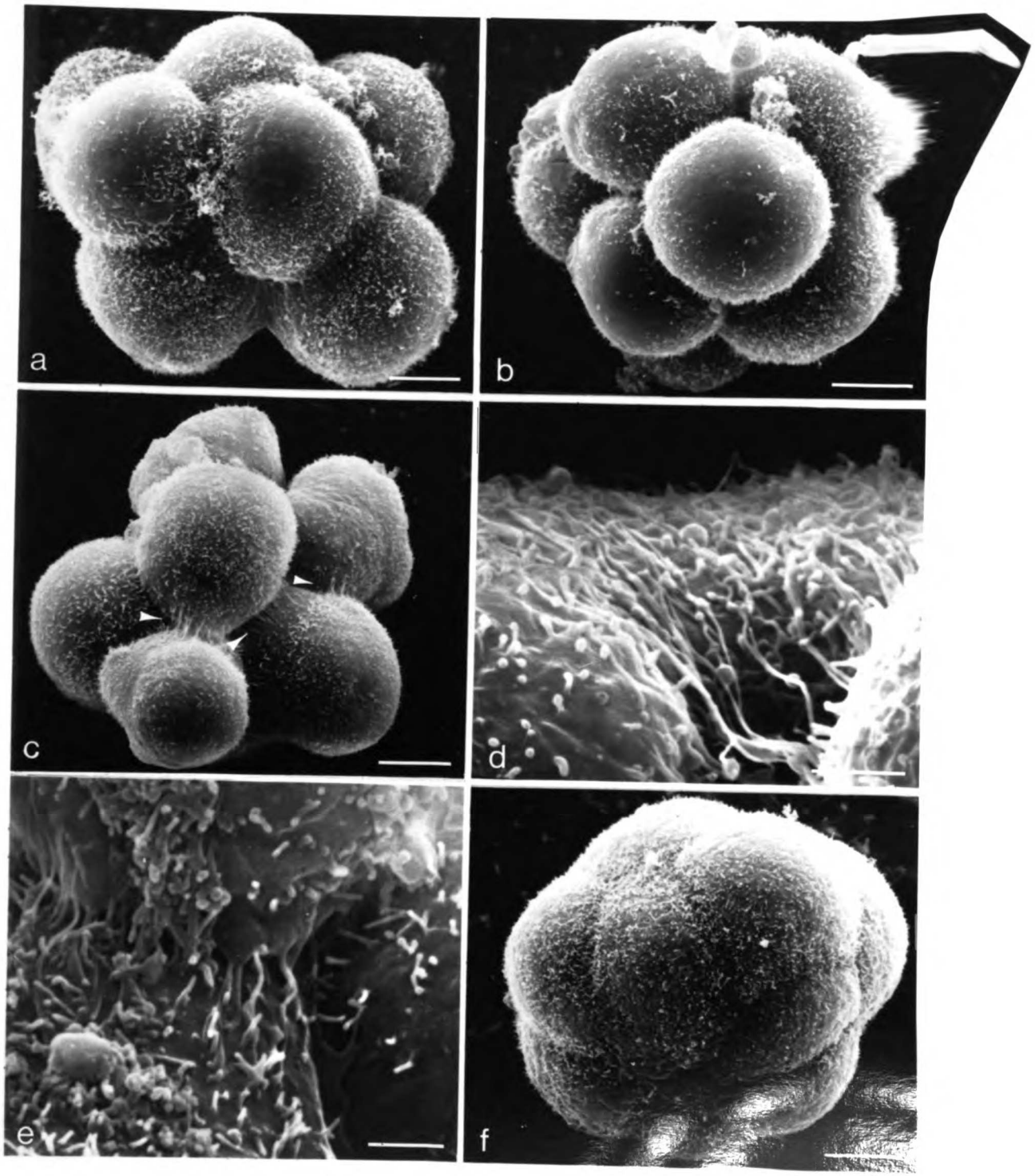
2-5). Scanning electron micrographs of originally uncompacted embryos treated 4.0 (**b**,**c**) and 10.0 (**a**,**d**) hours with 5μg/ml colcemid. **a**. An 8-cell embryo treated with Pronase prior to a 10-hour colcemid incubation. The blastomeres have not flattened, and no apical polarization of microvilli is evident. **b**. An 8-cell embryo treated 4 hours with colcemid. Compaction has not yet begun in this embryo, and surface microvilli are not polarized. **c**. An 8-cell embryo incubated 4.0 hours in colcemid. Compaction is almost complete in this embryo, and surface microvilli are polarized (P). **d**. An 8-cell embryo incubated 10.0 hours in colcemid. This embryo is completely compacted, and shows apical polarization of the surface microvilli (P). Scale bar = 10 μm.


a
b
p
p
c
p
p
d

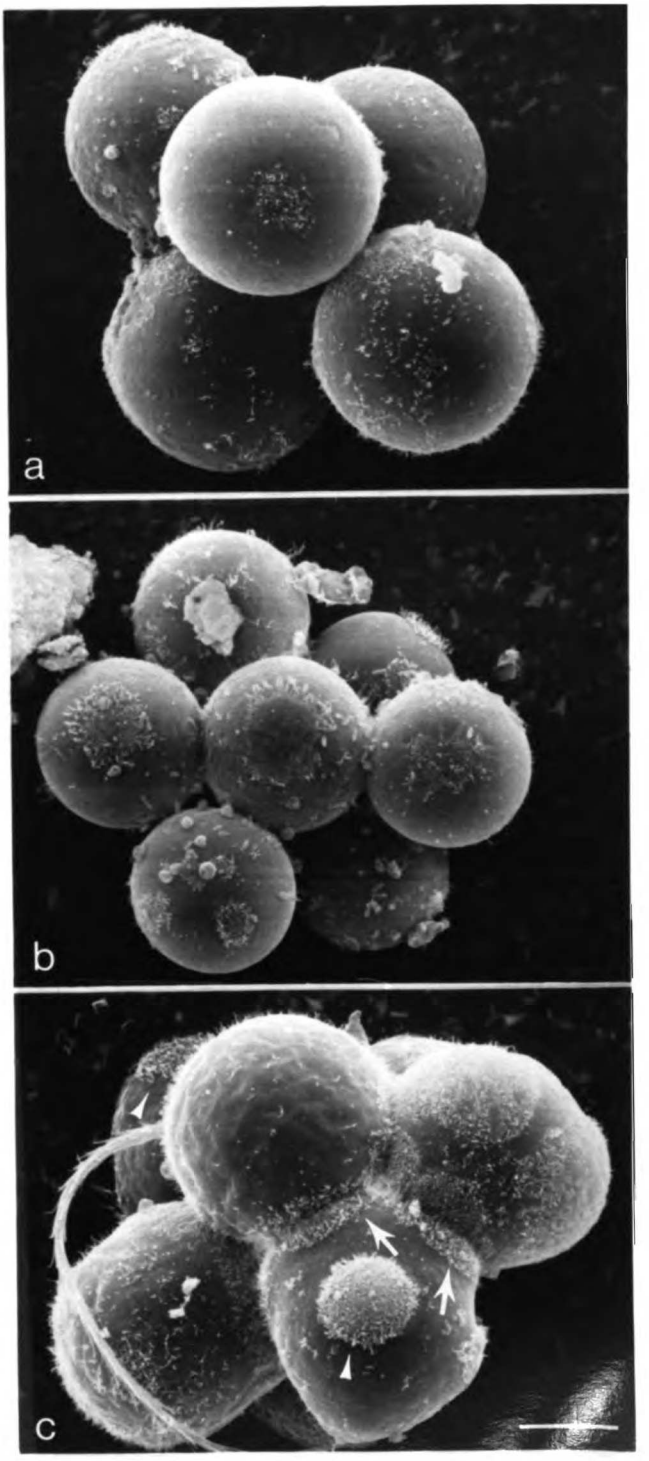
2-6). Scanning electron micrographs of originally compacted embryos treated 4.0 (**a**,**b**) and 10.0 (**c**,**d**) hours in 5 μg/ml colcemid. **a** and **b**. After 4.0 hours in colcemid, compacted embryos are partially decompacted, and surface microvilli are no longer obviously polarized. **c** and **d**. After 10.0 hours in colcemid, the embryos are completely decompacted, and have fewer microvilli than controls. These microvilli are often localized away from the apical pole of the blastomere (arrows), leaving the apical region relatively bare. After 20 minutes recovery, some microvilli can be seen stretching between blastomeres in the lateral regions (arrowheads, **c**). Scale bar = 10 μm.


a
b
c
d

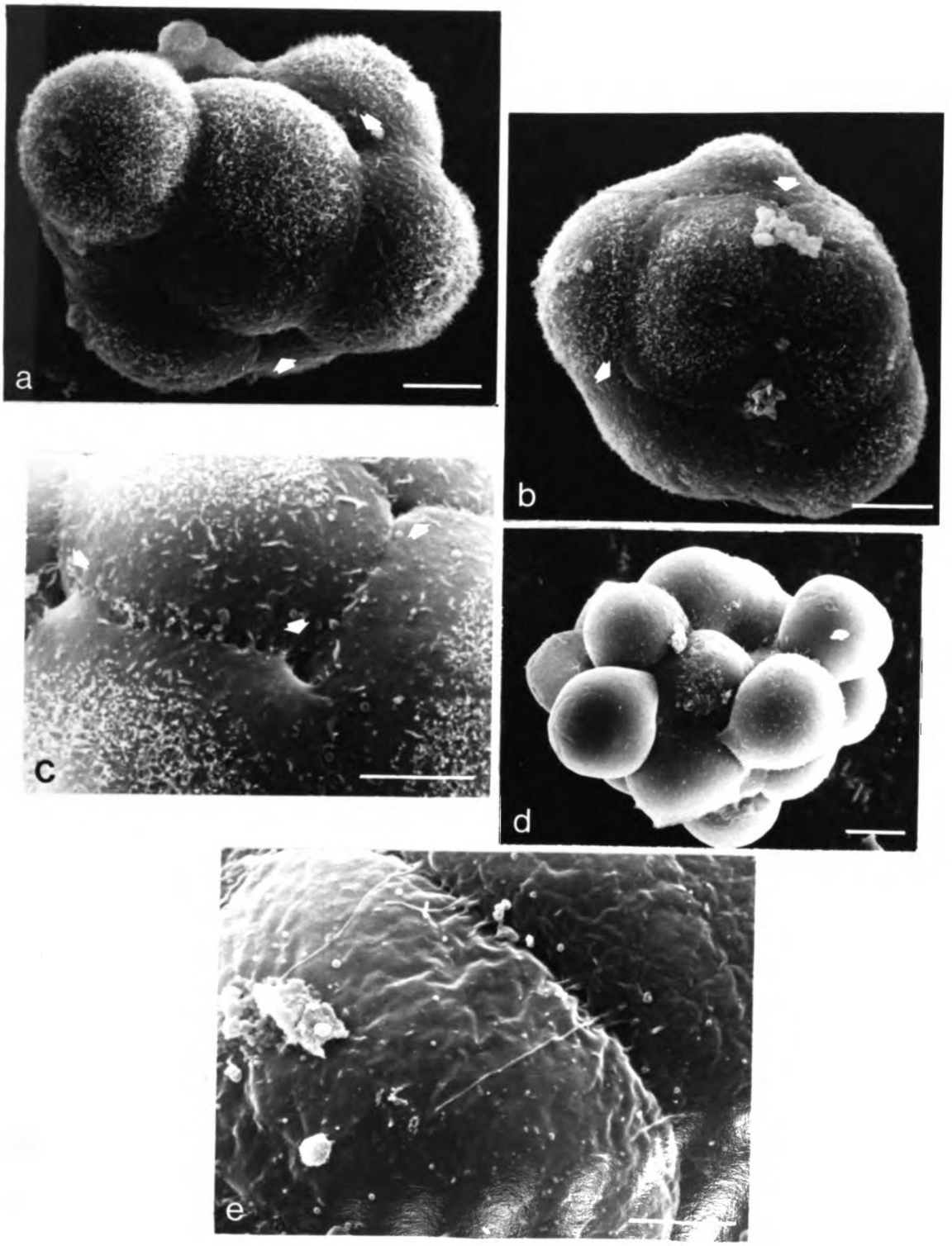
2-7). Scanning electron micrographs of embryos cultured 24 hours in 5 µg/ml colcemid. **a**. Originally uncompacted embryo. **b**. Originally compacted embryo. The embryos shown in **a** and **b** show that after 24 hours in colcemid, both of these classes of embryos are morphologically similar. The embryos are decompacted and appear to have fewer microvilli. Polarization of microvilli is no longer apparent, and blastomeres often have bare apical regions, as was seen in Figure 2-5. Scale bars = 10 µm. **c**. After 20 minutes recovery, microvilli are seen stretching between blastomeres (arrowheads). Scale bar = 10 µm. **d** and **e**. The lateral microvilli stretching between blastomeres take on several different morphologies, sometimes long and sinuous (**d**) and sometimes short and taut (**e**). Scale bars = 2 µm. **f**. An embryo cultured 24 hours from the 2-cell stage in 5 µg/ml ß-lumicolchicine. This embryo is at the 16-cell stage, shows close apposition of blastomeres, and an even distribution of microvilli on the free surface of the blastomeres. Scale bar = 10 µm.


a
b
c
d
e
f

2-8). Scanning electron micrographs of embryos cultured 24 hours in a combination of 5 μg/ml cytochalasin B and 5 μg/ml colcemid. **a** and **b**. Embryos are completely decompacted, and are morphologically similar to those cultured in cytochalasin B alone; surface microvilli are randomly localized in clumps on the surface of the blastomeres. **c**. After 20 minutes of recovery in normal medium, the blastomeres show increased intercellular apposition and prominent localization of surface microvilli in the regions of intercellular apposition (arrows). Increased numbers of microvilli are also seen elsewhere on the surface, but are still localized in large clumps (arrowheads). Scale bar = 10 μm.


a
b
c

2-9). Scanning electron micrographs of embryos cultured 24 (**a, b, c**) and 48 (**d, e**) hours from the 2-cell stage in 0.5 μg/ml tunicamycin. After 24 hours, embryos are at the 8-cell stage, and they undergo compaction and polarization. However, gaps in intercellular apposition are apparent, especially in areas where three blastomeres come into contact (arrows in **a, b, c**). Intercellular contacts are made through loboform (**c**) or lamelliform (**b**) protrusions. **d** and **e**. After 48 hours in tunicamycin, embryos contain 12-20 cells, intercellular apposition is decreased, and surface microvilli are almost absent. Intercellular connection are still maintained, through loboform and filiform protrusions (**e**). Scale bars = 10 μm (**d**), and 2 μm (**e**).


a
b
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e

Chapter Three

Summary

Mouse hatched blastocysts cultured *in vitro* will attach and form outgrowths of trophoblast cells on appropriate substrates, providing a model for implantation. Immediately after hatching, the surfaces of blastocysts are quiescent and are not adhesive. Over the period 24-36 hours post-hatching, blastocysts cultured in serum-free medium become adhesive and attach and spread on the extracellular matrix components fibronectin, laminin, and collagen type IV in a ligand specific manner. Attachment and trophoblast outgrowth on these substrates can be inhibited by addition to the culture medium of an antibody, anti-ECMr (anti-extracellular matrix receptor), that recognizes a group of 140 kD glycoproteins similar to those of the 140 kD extracellular matrix receptor complex (integrin) recognized in avian cells by CSAT and JG22 monoclonal antibodies. Addition to the culture medium of a synthetic peptide containing the Arg-Gly-Asp tripeptide cell recognition sequence of fibronectin inhibits trophoblast outgrowth on both laminin and fibronectin. However, the presence of the peptide does not affect attachment of the blastocysts to either ligand. Immunoprecipitation of ^{125}I surface labelled embryos using anti-ECMr reveals that antigens recognized by this antibody are exposed on the surfaces of embryos at a time when they are spreading on the substrate, but are not detectable immediately after hatching. Immunofluorescence experiments show that ECMr antigens and the cytoskeletal proteins vinculin and talin are enriched on the cell processes and ventral surfaces of trophoblast cells in embryo outgrowths, in patterns similar to those seen in fibroblasts, and consistent with their role in adhesion of the trophoblast cells to the substratum.

Materials and Methods

Antisera and Peptides

Antibodies used included rabbit anti-fibronectin (anti-Fn; Cappel Laboratories, Cochranville, PA), rabbit anti-laminin (anti-Ln; gifts of Dr. Deborah Hall, University of California, San Francisco, and Dr. Hynda Kleinman, National Institutes of Health), rabbit anti-heparan sulfate proteoglycan (anti-HSPG; gift of Dr. Hynda Kleinman), affinity purified rabbit antibodies to laminin and type IV collagen (anti-Col IV) (purchased from Dr. Heinz Furthmayr, Yale University; affinity purified anti-Ln was also the gift of Dr. Roy Ogle, University of Virginia) and goat anti-ECMr (formerly called anti-GP140; Knudsen et al., 1981). Anti-ECMr was produced in a goat by Dr. Karen Knudsen against a purified glycoprotein fraction of baby hamster kidney (BHK) cells, that consisted predominantly of a group of cell-substratum adhesion proteins of M_r 120,000-160,000 (Knudsen et al., 1981; Damsky et al., 1982). All of the above antibody preparations were used as purified IgG fractions; anti-ECMr was also used as an antiserum in some experiments. Rabbit antiserum against the cytoskeleton associated protein talin (Burridge and Connell, 1983) purified from chicken gizzard, was a gift of Dr. Mary Beckerle, University of Utah, and Dr. Keith Burridge, University of North Carolina. Monoclonal antibodies to vinculin (anti-Vnc) were purchased from Miles Scientific (Naperville, IL). Anti-fibronectin receptor (anti-FNR) antiserum was a gift of Drs. Erkki Ruoslahti and Michael Pierschbacher (La Jolla Cancer Research Foundation). Anti-entactin antiserum was a gift of Dr. Albert Chung (University of Pittsburgh). In order to remove any contaminating anti-Ln antibodies from the anti-entactin, 100 µl of a 1:10 dilution of anti-entactin serum was absorbed on one well of a 96-well plate that had been coated with 100 µl of 25 µg/ml Ln for 2 hours and rinsed three times with PBS containing 0.2% bovine serum albumin (BSA). The absorbed

serum retained its activity for entactin, as assessed by immunoblot analysis, and was used in outgrowth experiments without further dilution.. Purified laminin (Ln) was a gift of Dr. Deborah Hall, while purified fibronectin (Fn) and collagen type IV (Col IV) were purchased from Collaborative Research, Lexington, MA. Purified vitronectin was a gift of Drs. Erkki Ruoslahti and Michael Pierschbacher.

Two different types of synthetic hexapeptides were used in the experiments described below: 1. Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP). This hexapeptide contains the tripeptide cell binding region of fibronectin, and is active in preventing the adhesion of cultured cells to fibronectin (Pierschbacher and Ruoslahti, 1984a; Yamada and Kennedy, 1984; Ruoslahti and Pierschbacher, 1986). 2. Gly-Arg-Gly-Glu-Ser-Pro (GRGESP). This hexapeptide contains one amino-acid substitution relative to the first hexapeptide, and does not prevent the adhesion of cultured cells to fibronectin (Pierschbacher and Ruoslahti, 1984b). The peptides used were obtained from two different sources, from Peninsula Laboratories, Belmont, California, and as gifts from Drs. Michael Pierschbacher and Erkki Ruoslahti (La Jolla Cancer Research Foundation). Peptides were used at 250 and 500 μg/ml.

Embryo Culture

Female ICR mice (12 weeks old, Harlin) were superovulated by injection with 5 IU of pregnant mare's serum (Teikoku Hormone Mfg. Co., Japan) followed after 48 hours by an injection of 2.5 IU of human chorionic gonadotropin (hCG, Sigma) and were then caged with ICR males, and inspected the next morning for the presence of a vaginal plug, indicating insemination. Embryos were collected at the 2-cell stage (48 hours post-hCG) and cultured to the desired stage in microdrops (15-20 μl) of embryo culture medium (Biggers et al., 1971; Spindle, personal communication). Incubation of culture drops is under a layer of mineral oil, at 37° C, in a humidified atmosphere of 5% CO2 in

air. Embryos were routinely cultured *in-vitro* to the blastocyst stage instead of being flushed from the uterus at the blastocyst stage, since a much greater number of embryos is obtained this way.

Cell Culture

Mouse mammary tumor epithelial cells (MMTE) and human trophoblast cells (JAR) were generously provided by Dr. Caroline Damsky. MMTE cells were cultured in tissue culture flasks or 100 mm plates in DME H-21 medium (UCSF Cell Culture Facility) supplemented with insulin, gentamycin, penicillin-streptomycin, and 10% fetal calf serum. Fresh glutamine was added to this medium after two weeks. JAR cells were cultured in tissue culture flasks or 100 mm plates in DME H-21 supplemented with penicillin-streptomycin and 10% fetal calf serum. All cells were cultured at 37° C in a humidified atmosphere of 5% CO_2 in air.

Cinemicrography

Embryos were placed in a microdrop of culture medium (BME medium for hatched blastocysts) under oil in a Falcon 35 mm tissue culture dish which had been equilibrated at 37° C for four hours. The cover of the dish was inverted and two holes bored into opposite sides; one a pinhole, the other large enough to accept a syringe tip. The cover was then sealed onto the dish using high vacuum grease (Dow Corning). A piece of tubing was inserted into the large hole in the cover, and attached at at the other end to a tank containing 5% CO2 in air. The flow of gas was adjusted to a level that did not disturb the surface of the oil.

The chamber was then placed into a stage heater (Steier, 1975) controlled by a proportional temperature controller (Yellow Springs, model 72) on the stage of a Nikon Diaphot inverted microscope outfitted with Hoffman Modulation Contrast optics. The

embryos were filmed using a 10X or 20X objective, and their development recorded on Kodak Tri-X reversal film with a Bolex H-16 camera controlled by a Nikon CFX intervalometer. Frames were taken at 30-second intervals.

Experimental Incubation of Preimplantation Embryos

Dilutions of either anti-Ln, anti-CIV, or preimmune rabbit serum were made in embryo culture medium, then placed as microdrops under oil, and equilibrated in the incubator for 45 minutes. Two-cell embryos were placed in these drops, and cultured for 72 hours.

For incubation of preimplantation embryos in the presence of soluble extracellular matrix molecules, laminin and collagen type IV were diluted to a concentration of 10 µg/ml in embryo culture medium. Zona-free embryos were cultured from the 2-cell stage in microdrops of these dilutions. Alternatively, 35 picoliters of either laminin- or collagen type IV-containing medium was injected by pressure injection just under the zona pellucida of 2-cell embryos, into the perivitelline space, and the embryos were cultured 72 hours in drops of the same medium that was used for injection.

Outgrowth on Substrates of ECM Molecules

Antibody and peptide dilutions were prepared either in Eagle's Basal Medium (BME) supplemented with 4 mg/ml bovine serum albumin (BSA) and 1% Nutridoma-HU (Boehringer Mannheim Biochemicals) (modified from Spindle, 1980); or in CMRL medium 1066 (Gibco) supplemented with 0.5 mg/ml calcium lactate, 0.05 mg/ml sodium pyruvate, 4 mg/ml BSA (Sigma), and 1% Nutridoma-HU (Armant et al., 1986b). All media (with or without antibody or peptide) were filter-sterilized using

syringe-tip filters of 0.45 μm pore size (Millipore, HV) as they were loaded into the previously prepared wells (see below).

Substrates for experimental culture were prepared essentially as described previously (Giancotti et al., 1985) in 96-well tissue culture dishes with flat bottom wells (Falcon, #3075). Coating solutions used were: Ln (25 or 50 μg/ml), Fn (10 or 25 μg/ml), Col IV (10 or 25 μg/ml) or BSA (25 μg/ml). The coated wells were rinsed twice with sterile PBS, once with medium, then filled with the appropriate antibody or peptide dilution for the experiment and equilibrated at 37° C for 30-60 minutes.

Hatched blastocysts (144 hours post-hCG) were collected and rinsed in medium in agarose-coated dishes, then placed into the previously prepared wells, at 10-30 embryos per well, and incubated for 48-72 hours. Outgrowth was examined at various times during the culture period with a Nikon Diaphot inverted microscope, and photographed using Kodak Tri-X film. Embryo attachment in the absence of spreading was quantified by gently blowing a small amount of medium on each embryo, using a glass pipette pulled to a very fine bore. Those that did not move were considered to be attached.

The extent of spreading data were obtained by photographing embryos at a magnification of 20X, printing each negatives the same size, and then measuring the area of each outgrowth from the prints using a Numonics digitizer. The final value for each embryo was calculated as the average of three tracings, and the final value for each treatment was calculated as the average of the values from at least 11 embryos. .

Immunofluorescence

A. Preimplantation Embryos

Indirect immunofluorescence assays were performed on live or 2% p-formaldehyde-fixed preimplantation embryos. Zona-free live embryos were incubated for 30 minutes in 20 µl drops of antiserum diluted 1:40 in embryo culture medium. Following three rinses in culture medium to remove unbound primary antibody, embryos were incubated for 15 minutes in fluorescein- or rhodamine-conjugated secondary antibody (goat anti-rabbit, rabbit anti-goat, or rabbit anti-sheep; Cappel) diluted 1:100 in phosphate buffered saline, and subsequently rinsed three times in culture medium. Embryos were then mounted in a drop of medium, examined with a Zeiss fluorescence microscope, and photographed on Kodak Ektachrome 400 or Tri-X film.

Zona-free embryos were fixed for 45 minutes in 2% formaldehyde (made fresh from paraformaldehyde and diluted 1:1 in 0.2M phosphate buffer) followed by two 5-minute rinses. Following fixation, some embryos were permeablized by treatment with 1% NP40 in PBS. All embryos were incubated for 30 minutes in 20 µl drops of antiserum diluted 1:40 in 0.1M phosphate buffer. Following five 5-minute rinses in 0.1M phosphate buffer, embryos were incubated for 15 minutes in fluorescein- or rhodamine-conjugated secondary antibody (as above), then rinsed a further five times (5 minutes each time). The embryos were then infiltrated with glycerol (50% in 0.1M phosphate buffer), mounted in a drop of 50% glycerol, examined and photographed as above.

B. Embryo Outgrowths

Circular glass coverslips (Fisher, 12mm) used in the following experiments were coated with Fn (10 µg/ml) at the concentrations described above for 96-well

plates. After coating, the coverslips were placed into 35 mm petri dishes (Falcon, #1008; five coverslips per dish), covered with medium, and 10-20 hatched blastocysts (144 hours post-hCG) placed on each coverslip.

After 48-72 hours culture, embryo outgrowths were rinsed three times in warm (37o C) PBS and then fixed for 30 minutes in freshly prepared 2% paraformaldehyde in PBS (pH 7.3). Embryos were reacted with 0.15 M glycine in PBS for 15 minutes to block free aldehyde groups, as described above for the hatched blastocysts. They were then permeabilized with a 3-minute treatment in acetone at -20° C, and subsequently incubated in PBS containing 0.5% BSA to prevent non-specific antibody binding. Following several rinses in PBS, the outgrowths were incubated for 60 minutes in either anti-ECMr, anti-FNR, anti-Vnc, anti-talin, or non-immune antibodies (or combinations of two of the above for co-localization experiments). The embryos were then rinsed five times in PBS and incubated in fluorescein- and/or rhodamine-conjugated secondary antibodies (Cappel) for 60 minutes, then rinsed a further five times in PBS, and mounted on slides in mounting medium containing p-phenylene diamine to prevent quenching of the fluorescent signal. Embryos were examined with a Zeiss Universal microscope outfitted for epifluorescence, and photographed using Kodak Tri-X film.

Radiolabelling of Cells and Embryos

Cultured cells [mouse mammary tumor epithelial cells (MMTE), or human trophoblast cells (JAR)], hatched blastocysts (144 hours post-hCG), and embryo outgrowths (216 hours post-hCG) were surface labelled with ^{125}I using the lactoperoxidase-glucose oxidase catalyzed radioiodination method. Both the cultured cells and the hatched blastocysts were labelled in suspension. Cells were harvested using 2mM EDTA, 0.5% BSA in PBS, rinsed three times with 20mM glucose in PBS (PBS-

Glucose), then resuspended in 1 ml of the same buffer. Lactoperoxidase (50μg; Boeringer-Mannheim Biochemicals), glucose oxidase (50 U; Sigma), and Na125I (400 μCi; Amersham) were then added and the reaction was allowed to proceed, with mixing, for 10 minutes on ice. After labelling, cells were rinsed three times in Dulbecco's modified Eagle's medium (DME; UCSF Cell Culture Facility) and resuspended in one ml of DME. For radiolabelling of hatched blastocysts, a group of 300 hatched blastocysts was suspended in 200 μl of PBS-Glucose, and lactoperoxidase, glucose oxidase, and Na125I were added. The iodination reaction was allowed to proceed for 10 minutes on ice, and the embryos were then rinsed three times in DME and resuspended in 200 μl of DME. For radiolabelling of embryo outgrowths, a group of 200 72-hour embryo outgrowths in one well of a 96-well plate (Costar) was rinsed six times with PBS-Glucose, then left in a final volume of 200 μl of the same buffer. Lactoperoxidase, glucose oxidase, and Na125I were added and reacted with the embryos for 10 minutes on ice. The outgrowths were then rinsed 10 times with DME and left in a final volume of 200 μl.

Immunoprecipitation

For immunoprecipitation of radiolabelled proteins, either normal goat or anti-ECMr antibodies were added to the intact labelled cells, hatched blastocysts, and embryo outgrowths. In the case of outgrowths, 10 μg of cold Fn was added to the mixture to reduce non-specific precipitation of Fn substrate labelled during the iodination of the attached outgrowths. All samples were then incubated on ice for one hour, rinsed three times with PBS, and lysed for 30 minutes in lysis buffer (200 mM octylglucoside, 1 mM PMSF in PBS) with frequent agitation. Cell or embryo lysates were spun for 20 minutes in the cold at 12,000 rpm to pellet nuclei and other insoluble components, and the resulting supernatant was precleared twice on 100 μl pellets of Sepharose 4B (Pharmacia) beads for 30 minutes at 4o C with mixing. The precleared supernatant was then immunoprecipitated on a 100 μl pellet of Protein A-Sepharose 4B (Pharmacia)

beads for 1 hour at 4° C with mixing. The beads were pelleted with a low-speed spin, and washed with a series of buffers: 1. Tris-NP40 buffer (0.05 M Tris pH 8.0, 0.4 M NaCl, 0.005 M EDTA, 1% NP40) (TNB). 2. TNB with 0.1% SDS. 3. TNB with 1.0 M NaCl. After washing, the beads were pelleted and resuspended in electrophoresis sample buffer without 2-mercaptoethanol, and the immunoprecipitated proteins solubilized by heating the sample at 100° C for 3 minutes. In some experiments, the surface labelled embryos and cells were lysed and spun for 10 minutes in a microfuge before a one-hour incubation with the primary antibodies. Following primary antibody incubation, the lysates were precleared with sepharose and immunoprecipitation was performed as described above.

Polyacrylamide Gel Electrophoresis

Immunoprecipitates were analyzed following the method of Laemmli (1970) using 7.5% polyacrylamide slab gels containing SDS. Prestained molecular weight markers (BRL) were used, and consisted of myosin (200 kD), phosphorylase b (97.4 kD), and BSA (68 kD). The gels were stained with Coomassie Brilliant Blue, fixed, dried down, and exposed to X-ray film (Kodak XAR-5).

Results

Extracellular Matrix in Preimplantation Development

Because extracellular matrix molecules have been found to be synthesized in the early mouse embryo before the first basement membrane is organized (Cooper and MacQueen, 1983), it is possible that these molecules may also have some role in compaction and blastocoel formation. To examine this possibility, two types of experiments were performed: 1.) Embryos were incubated in culture medium containing antibodies to Ln or Col IV; 2.) Medium containing Ln or Col IV was microinjected into the perivitelline space of two-cell embryos, and these embryos were then incubated in the same medium to the blastocyst stage.

Two-cell embryos were incubated for 72 hours in various dilutions (1:10 to 1:100) of anti-Ln and anti-CIV. As a control, 2-cell embryos were incubated in medium containing preimmune antibodies at the same dilutions. Neither anti-Ln nor anti-CIV had any effect on development at any dilution; embryos cultured in the presence of either antibody underwent cleavage, compaction, and blastocoel formation at the same rate and in the same proportion as did embryos cultured in the presence of preimmune antibodies.

As another way of determining whether Ln or CIV play a role in early development, embryos were incubated in medium containing either Ln or CIV in soluble form. These experiments were done in two ways. In the first method, 2-cell embryos were treated with Pronase to remove their zonae pellucidae, and then incubated, zona-free, in medium containing either 10 μg/ml Ln or 10 μg/ml CIV. In the second method, 35 picoliters of medium containing 10 μg/ml Ln or 10 μg/ml CIV was microinjected underneath the zona pellucida and into the perivitelline space of 2-cell embryos. These

embryos were then incubated for 72 hours in a drop of the same medium. Embryos from either method underwent cleavage, compaction, and blastocoel formation at the same time as controls; there was no effect on development of either soluble Ln or soluble CIV.

Indirect immunofluorescence was performed on both live and fixed and permeablized zona-free embryos using anti-Ln, anti-CIV, and anti-HSPG in order to examine the distribution of these components in the early mouse embryo. Diffuse intracellular staining for Ln and HSPG was detectable beginning at the 16-cell stage (not shown), and some equivocal staining for Ln, HSPG, and CIV was detectable at the 8-cell stage. No staining for Ln, CIV, or HSPG was detectable at earlier stages.

Normal Outgrowth Formation by Hatched Blastocysts

Blastocyst outgrowth in culture is asynchronous among embryos and occurs following hatching of the embryo from its zona pellucida. Immediately after hatching, blastocysts are not adhesive and when viewed by time lapse cinemicrography their surfaces are quiescent (Figure 3-1a). After 10-12 hours in culture in serum-containing medium, the trophoblast cells of most embryos show signs of protrusive activity (Figure 3-1b). Spreading is first detectable in the faster-developing embryos after 15 hours in culture (Figure 3-1c), and becomes extensive after 20-24 hours; other embryos have yet to begin spreading at this time (Figure 3-1d). By 36 hours in culture, the slower-developing embryos have also begun to spread, and by 48 hours all have formed extensive outgrowths. This sequence of events is similar when the embryos are grown in the absence of serum, however, the time span over which it occurs is more extended. Embryos cultured in the absence of serum have begun to spread after about 36 hours, and have formed extensive outgrowths by 72 hours in culture.

The timing of outgrowth formation was similar for embryos cultured in serum-free medium on either Fn, Ln, but seemed slightly slower on Col IV. Most embryos had attached and begun to spread on these substrates after 36 hours, and reached maximal spreading after 48-60 hours (Figure 3-2a, b, c). Identical results were obtained for all substrate coating concentrations used. Embryos cultured on a substrate of BSA in serum-free medium did not attach or spread (Figure 3-2d).

Specificity of Blastocyst Interactions with Defined Ligands

The interactions of embryos with Fn, Ln, and Col IV were ligand specific; embryos attached and spread on Fn in the presence of non-immune antibodies or anti-Ln, but not in the presence of anti-Fn (Figures 3-3 and 3-5). Outgrowth on Fn was not due to the presence of small amounts of vitronectin (a possible contaminant of Fn preparations and therefore of anti-Fn sera), since attachment and spreading on vitronectin substrates were unaffected by the presence of anti-Fn antibodies (results not shown). Embryos attached and spread on Ln in the presence of either non-immune antibodies or anti-Fn but not in the presence of anti-Ln (Figures 3-3, 3-4, and 3-5). Embryo outgrowth was also unaffected by the presence of antibodies against Col IV (Figures 3-4 and 3-5) and against entactin (not shown), two common contaminants of Ln preparations. Finally, blastocyst adhesion and spreading on Col IV are inhibited by anti-Col IV antibodies, but not by non-immune antibodies or anti-Ln antibodies (Figures 3-4 and 3-5).

Effects on Blastocyst-Ligand Interactions of Anti-ECMr Antibodies and Synthetic Peptides

Anti-ECMr, a polyclonal antibody made against 120-160 kD adhesion glycoproteins isolated from hamster fibroblasts, completely inhibited both attachment and outgrowth of embryos on Fn, Ln (Figures 3-6 and 3-7) and Col IV (not shown). The

effects of these antibodies were reversible; embryos removed from anti-ECMr-containing medium after 48 hours culture, rinsed and put back into control medium began to spread within 4 hours, and formed outgrowths within 12 hours (Figure 3-6).

Peptides from two different sources (see Materials and Methods) were used to examine the role of the Arg-Gly-Asp-containing cell-recognition site in the formation of embryo outgrowths. Results were similar for peptides from both sources. Neither the active (GRGDSP) nor the control (GRGESP) peptide at concentrations as high as 0.5 mg/ml prevented attachment of embryos to substrates coated with 10 μg/ml Fn. However, GRGDSP had a pronounced effect on trophoblast outgrowth; both the incidence and extent of outgrowth on Fn was greatly reduced (Figures 3-8, 3-9, and 3-10). Embryo outgrowth on Fn in the presence of GRGESP was similar to that seen in the absence of peptide, both in incidence and extent (Figures 3-8, 3-9, and 3-10).

For embryos on Ln substrates, a similar effect was seen. Again, neither peptide prevented attachment of blastocysts to Ln, but both the incidence and extent of trophoblast outgrowth were reduced in the presence of GRGDSP (Figures 3-8, 3-9, and 3-10). In contrast to its lack of effect on outgrowth on Fn, the control peptide, GRGESP, also had some effect on embryo outgrowth on Ln, although to a lesser extent than did GRGDSP (Figures 3-9 and 3-10).

Synthesis and Expression of Adhesion Receptors by Embryo Outgrowths

The goal of these experiments was to determine, by immunoprecipitation and immunofluorescence, whether mouse trophoblast cells produce 140kD antigens similar to those synthesized by cultured cells, and whether these antigens are expressed at a time and location consistent with their proposed function in outgrowth formation. Two different cell lines were examined; mouse mammary tumor epithelial cells (MMTE), and

human trophoblast cells (JAR). Two batches of embryos were also examined: 1) embryos surface-labelled with ^{125}I at the hatched blastocyst stage, and 2) embryos cultured 72 hours on a Fn substrate, then surface-labelled with ^{125}I. Immunoprecipitation of labelled cell-surface proteins was then carried out, either by adding the antibody to lysates of surface-labelled cells or embryos, or by incubating the intact cells or embryos in antibody prior to lysis. The immune complexes were then precipitated using protein A-Sepharose. Figure 3-11, panel A, shows the pattern precipitated when anti-ECMr was exposed to the surfaces of labelled intact embryos and cells. Two bands are recognized on the trophoblast surface of embryo outgrowths: one at about 144 kD, and the other more diffuse, at about 120-125 kD (Lane A1). This pattern is similar to that recognized on intact MMTE, although the lower band is more diffuse and migrates slightly more slowly in the latter (125-135 kD; Lane A2). When embryos that had been labelled with ^{125}I at times during the first 24 hours after hatching were precipitated, no distinct bands in the 120-160 kD region of the gel were recognized above the background (Lane A3), although a diffuse band at about 80 kD was detected.

When surface-labelled embryos and cells were lysed prior to exposure to anti-ECMr (Figure 3-11, panel B), the patterns were somewhat more complex. In addition to the two bands recognized previously, bands at about 200 kD and 94 kD were precipitated by anti-ECMr from the embryo outgrowths (Lane B1). A band at about 200 kD is also recognized on the MMTE cells under these conditions (not shown). On JAR cells, anti-ECMr recognizes bands at 120 kD and 150 kD (Lane B2), which are likely to be analogous to the 120 kD and 140 kD bands in the embryo outgrowths. Anti-ECMr also recognizes a band at 200 kD in JAR cells, as well as an additional band at about 180 kD, suggesting that it has a more complex array of ECMr-related components than the mouse trophoblast.

These results indicate that mouse embryo outgrowths display ECMr related antigens at the cell surface when they are capable of interacting with the substrate. Furthermore, the components recognized on the outgrowths are similar to those recognized by anti-ECMr on cultured cells of the same species (MMTE). They are similar as well, to the components recognized on a human trophoblast cell line by anti-ECMr, although the pattern in the JAR cells contains an additional component at 180 kD.

To determine the distribution and organization of ECMr antigens in embryos, hatched blastocysts and embryo outgrowths were stained with anti-ECMr and an antibody against the human FN receptor (anti-FNR). Like anti-ECMr, the antiserum against the FNR recognizes the ß chain of the ß1 family of integrin. Previous studies on murine melanoma cells using anti-ECMr and anti-FNR showed that both antibodies immunoprecipitate components at 120 and 140 kD and immunoblot the 120 kD component (Kramer and Damsky, 1986; Kramer et al., submitted). The distribution patterns of these two antibodies were correlated with those of the cytoskeletal proteins talin (Burridge and Connell, 1983), and Vnc (Geiger, 1979), which are components of cell-matrix contact sites (adhesion plaques) of cultured cells (Burridge and Feramisco, 1980; Geiger, 1979; Burridge and Connell, 1983). Indirect immunofluorescence was performed on several groups of embryos: hatched blastocysts, and hatched blastocysts cultured 48 and 72 hours post-hatching on Fn substrates.

The embryo outgrowths are highly three dimensional with a rounded inner cell mass (ICM) and a surrounding flattened area of trophoblast outgrowth (Figure 3-12a). By focusing at different optical planes of this structure, the distribution pattern of the ECMr antigens and the cytoskeletal antigens Vnc and talin can be compared. In this study, the combination of antibodies that gave the best results in double indirect

immunofluorescence experiments was anti-FNR and anti-Vnc. Results from single staining experiments using anti-ECMr alone gave similar staining patterns as anti-FNR (e.g. compare Figures 3-12b with 3-13a, and 3-12g with 3-13g). At both early (48 hours post-hatching) and later (72 hours post-hatching) periods, anti-ECMr and anti-FNR staining were strongly enriched at the surface of cells in the top part of the ICM (Figure 3-12b). In contrast, staining for both Vnc (Figure 3-13b) and talin (not shown) in this region was diffuse and only slightly above the preimmune control. In the early outgrowth period, at the earliest time points that I could document, the pattern of cell-matrix contact sites was highly organized in the lamellopodial protrusions of the spreading trophoblast cells, as detected by staining with anti-Vnc (Figure 3-12c, d). Staining for anti-ECMr or anti-FNR in trophoblast cells was somewhat more diffuse. Double staining for FNR and Vnc showed extensive colocalization of discrete cell-matrix contact sites in these early outgrowths (Figure 3-12e, f). Examination of the cells at the periphery of later outgrowths shows that anti-ECMr continues to be enriched on filopodial protrusions. In areas where portions of the outgrowth have been torn away, anti-ECMr staining is seen on the substrate as a series of bright strips and dots (Figure 3-12g). Talin is also arrayed in streaks and spots in areas of trophoblast spreading (Figure 3-12h). In all of these samples the pattern of highly fluorescent patches is reminiscent of the staining patterns for avian integrin (the CSAT antigen complex) and for talin and Vnc on avian fibroblasts (Damsky et al., 1985).

In an effort to examine more carefully the organization of adhesion receptors and the cytoskeleton, FNR and Vnc were localized in three areas of a 72-hour outgrowth (Figure 3-13). As indicated above , when focusing near the top of the rounded inner cell mass, anti-FNR (and anti-ECMr) staining is strongly enriched at the cell surface, while Vnc (and talin) staining is diffuse and only slightly above background (Figure 3-13a, b). A group of cells lying on the embryo surface in a region intermediate to the ICM and

the trophoblast were photographed at two planes of focus: one near the apical regions of these cells and the other more basally. This group of cells has smaller nuclei than those at the periphery of the outgrowth and may represent endoderm (Gonda and Hsu, 1980). Both anti-Vnc and anti-FNR staining are strongly enriched at the cell surface and are largely co-extensive. The organization of Vnc changes dramatically in the more basal regions of these cells, becoming highly discrete and punctate. The pattern of anti-FNR also changes. It is enriched in the general areas of strong Vnc staining (see arrows, Figure 3-13), but is more diffuse. At the periphery of the outgrowth at the level of the substrate, a discrete pattern of contact sites is visible for both antigens, although the anti-FNR staining, like that of anti-ECMr, has additional diffuse staining.

In hatched blastocysts stained with either anti-ECMr or anti-FNR, no fluorescent signal above the control level was seen (not shown).

Discussion

The results described above make three major points. First, mouse blastocysts will specifically attach and spread on a variety of defined extracellular matrix glycoproteins including Fn, Ln and Col IV (Figures 3-2 to 3-5). Second, as described in Figures 3-6 and 3-7, the family of cell surface ECM receptors, variously called, in avian cells, the 140 kD complex (Akiyama et al., 1986), the CSAT antigen (Neff et al., 1982) and, more recently, integrin (Tamkun et al., 1986) is involved in all these blastocyst-ligand interactions. Third, the immunoprecipitation and immunofluorescence data (Figures 3-11 and 3-12) show that blastocysts synthesize and express 140kD integrin-like matrix receptors at a time and location consistent with their proposed role in blastocyst attachment and outgrowth.

ECM in Preimplantation Embryos

Previous studies have determined that some ECM components, notably laminin and heparan sulfate proteoglycan, are synthesized very early in development, before the formation of any organized basement membrane or stroma (Adamson and Ayers, 1979; Leivo et al., 1980; Cooper and MacQueen, 1983; Wu et al., 1983; Dziadek et al., 1985; Dziadek and Timpl, 1985). The possible function of these ECM molecules in these early stages is unknown, although in teratocarcinoma embryoid bodies, Ln appears to be involved in specifying the polarity of the outer epithelium (Grover et al., 1983). In order to determine whether laminin, or perhaps some other ECM component such as CIV, has an analogous function in early mouse development, embryos were incubated in the presence either of soluble Ln or CIV, or of antibodies to these two molecules. Neither of these two treatments had any detectable effect on development from the 2-cell stage to the expanded blastocyst stage. These results argue against any direct involvement of either Ln or CIV in early developmental events.

Blastocyst Interactions with Defined ECM Ligands.

Recognition of Fn, Ln and Col IV by blastocysts is ligand specific and not due to cross contamination among the ligands, since antibodies against the individual ECM components interfere only with blastocyst attachment to that particular ligand (Figures 3-2 to 3-5). These observations in part support those of Armant et al., (1986a), who reported that blastocysts could attach and spread on Fn and Ln. However, the lack of outgrowth on collagen types IV and I reported in those studies contrasts both with the data reported here (Figures 3-4 and 3-5) and with previous observations that the presence of a substrate derived from rat tail collagen could also promote embryo outgrowth in the absence of serum (Wilson and Jenkinson, 1974). The reasons for this discrepancy between my data and that of Armant et al. are not clear, but may lie in differences in the source of Col IV, in the nature of the plastic substrate, or in the method used to coat the substrates.

The invading trophoblast would be expected to encounter a variety of ECM ligands during implantation. In fact, both Fn and Ln have been found to be prominent components of the uterine basement membrane and stroma at the time of implantation (Wewer et al., 1985; Grinnell et al., 1982). Ln may be particularly significant in this regard, as it has been shown that uterine decidual cells in the vicinity of the implanting conceptus synthesize and surround themselves with a Ln-rich basal lamina (Wewer et al., 1985). At the same time, the amount of Fn in the uterine stroma near the implantation site decreases considerably (Grinnell et al., 1982).

Both Fn and Ln, and perhaps collagen as well, have more than one mechanism by which they can interact with the cell surface. In addition to the RGD-containing cell-binding domain (Pierschbacher and Ruoslahti, 1984a), Fn has a heparin-binding

domain (Hakomori et al., 1984) which may mediate cell attachment via heparin- or heparan sulfate-containing cell surface molecules (Saunders et al., 1986). Ln is reported to have at least two cell-binding regions, one near the intersection of the long and short arms (Liotta et al., 1987; Graf et al., 1987) and the other near the end of the long arm (Engvall, 1986).

Armant et al., (1986b) have reported that small peptides containing the cell-binding sequence of Fn (i.e. GRGDSP), at concentrations as low as 200 µg/ml, inhibit both blastocyst attachment and outgrowth on Fn, but not on Ln. In contrast to those results, my experiments show that the GRGDSP peptide at 250-500 µg/ml does not inhibit attachment to either Fn or Ln, but inhibits trophoblast outgrowth on both ligands. Recently Farach et al. (1987) have also reported that some outgrowth can occur on Fn in the presence of RGDS. I found that the GRGESP (control) peptide had no effect on blastocyst-Fn interactions, but had a slight effect on trophoblast outgrowth on Ln. The differences in the data on attachment from the two laboratories may be related to differences in the methods used to assess attachment in the absence of spreading. In the present study, a gentle stream of medium was blown against the embryos whereas Armant et al (1986a,b) report shaking the dish. The reason that I find an effect of these peptides on outgrowth on Ln while Armant et al. do not is more difficult to ascertain, but may reflect differences in the sources of Ln, or in methods of Ln purification. In my hands, antibodies against Col IV, entactin, and Fn do not inhibit outgrowth on Ln, arguing that these molecules, as possible contaminants of Ln preparations, are not responsible for the outgrowth of embryos on Ln.

There is precedent for an effect of RGD- and RGE-containing peptides on cell interactions with Ln. Madri et al., (1987) have reported effects of both RGD- and RGE-containing peptides on endothelial cell-Ln interactions. Grabel and Watts (1987) find

that GRGDSP peptides inhibit teratocarcinoma cell migration on Ln as well as on Fn, and Thiery et al. (1987) have reported that GRGESP affects migratory behavior of neural crest cells on Fn. The inhibition of trophoblast outgrowth on both Fn and Ln by GRGDSP is also consistent with equilibrium gel filtration experiments showing that the interactions of purified avian integrin with either Fn or Ln can be disrupted by small RGD-containing peptides (Horwitz et al., 1985). Taken together, these observations suggest that small peptides related to the cell recognition sequence of Fn, can have diverse effects depending on the cell type, the substrate and the biological activity being monitored.

The fact that in my hands, the active peptide affects embryo outgrowth on, but not attachment to, both Fn and Ln suggests that trophoblast is mediated by additional mechanisms. Farach et al., (1987) recently reported that soluble heparin inhibited mouse embryo attachment and outgrowth on both Fn and Ln to a greater extent than did RGDS, and that substrates of a heparin-binding protein, platelet factor 4, supported attachment but only limited outgrowth. These results, considered with those reported here, imply that outgrowth relies primarily on mechanisms that involve recognition of sequences related to the cell binding region of Fn, whereas attachment may be mediated through heparin- or heparan sulfate-containing moieties as well.

The Role of the Integrin Receptor Family in Blastocyst-ECM Interactions.

In their interactions with Fn, Ln, and Col IV, blastocysts appear to utilize a 140kD family of cell surface adhesion receptors, inasmuch as both trophoblast attachment and spreading on all three of these substrates are inhibited by anti-ECMr. The 140 kD receptor complex recognized in mouse blastocyts and other mammalian cells by anti-ECMr is very similar to the integrin complex identified by the CSAT and JG22 monoclonal antibodies in avian systems. Such similarity is demonstrated by several

criteria: Firstly, anti-ECMr, CSAT and JG22 all affect cell attachment to a broad range of ECM ligands including Fn, Ln and Col IV (Hall et al., 1987; Horwitz et al.,1985; Tomaselli et al., 1987); secondly, the constituent polypeptides of both the ECMr complex and avian integrin behave similarly on reduced and non-reduced gels (Knudsen et al., 1985; Tomaselli et al., 1987); thirdly, the anti-ECMr used in this study and an antibody against band 3 of the integrin complex (Buck et al., 1986) precipitate identical polypeptide patterns from labelled rat PC12 cells (Tomaselli et al., 1987); and finally, the anti-integrin band 3 antibody reacts in the immunoblot procedure with the lowest molecular weight band (120kD) immunoprecipitated from rat PC12 cells by anti-ECMr (Tomaselli et al., 1987). Gene cloning and protein sequencing data have shown in turn that the avian and mammalian adhesion receptor complexes are also closely related to the dimeric fibronectin receptor isolated using fibronectin affinity chromatography (Pytela et al., 1985a; Patel and Lodish, 1986). In addition, the anti-human FNR used in these studies and anti-ECMr recognize a similar pattern of polypeptides on murine melanoma cells (Kramer et al., submitted). These observations suggest that the mammalian adhesion receptors recognized by anti-ECMr in the mouse embryo are members of the large family of adhesion receptors that includes both the Fn receptor and the avian integrin complex. Embryo attachment to the broad range of substrates studied here may reflect the presence of several ligand-specific heterodimeric receptors. It is also possible, however, that the 120 and 140 kD components that comprise most of the anti-ECMr-reactive material constitutes, at least in part, a promiscuous receptor, capable of interacting with more than one ECM ligand. Such a receptor, in the ß1 integrin family has been proposed by Wayner and Carter (1987). The platelet adhesion receptor GP IIb-IIIa, a member of the ß3 subfamily of integrin has also been shown previously to act as a receptor for at least four ECM ligands, including fibronectin (Pytela et al., 1986).

Anti-ECMr inhibits attachment to a broad range of substrates, but does not inhibit all cell-matrix interactions: for example, it does not affect cell attachment to thrombospondin (Tuszynski et al., 1987) nor does it affect attachment of PC12 cells to polylysine or to the surfaces of muscle or neuronal cells (Tomaselli et al., 1987). Anti-ECMr inhibits both attachment and spreading of mouse blastocysts on defined substrates (Fn, Ln, and Col IV). Natural ECM is more complex, however, containing proteoglycans, glycosaminoglycans, and thrombospondin in addition to Fn, Ln, and collagens. Anti-ECMr does not inhibit blastocyst attachment to the complex ECM laid down by bovine corneal endothelial cells, but does inhibit trophoblast outgrowth on this matrix (Glass and Damsky, unpublished experiments). These observations stress the importance of integrin-like adhesion receptors in trophoblast outgrowth while demonstrating that blastocysts are able to interact with their surrounding ECM in more than one way.

Expression and Distribution of Integrin on Blastocysts

Immunoprecipitation of ^{125}I-labelled surface proteins from intact embryo outgrowths with anti-ECMr produces a set of proteins that are very much like those found in MMTE cells. These bands in mouse embryos have molecular weights of approximately 140 and 120 kD, similar to those in MMTE cells, but closer in molecular weight to each other than those found in human trophoblast cells (Figure 3-11). In addition, a band at about 200 kD and another at about 95 kD are precipitated from whole lysates of surface-labelled mouse embryo outgrowths. The 200 kD band is also precipitated from both the MMTE and JAR cells, and is of similar molecular weight in all three cell types. This band may be related to attachment to Ln or Col IV since it is not precipitated from JAR cells by a monoclonal antibody that inhibits JAR cell attachment to Fn only, whereas both the 120 kD and 150 kD bands are precipitated by that antibody (Damsky and Crowley, unpublished results). The 95 kD band was found only in embryo

outgrowths, and its relationship to the higher-molecular weight components is unknown. The fact that these two additional bands are precipitated from whole lysates but not from lysates of cells or embryos exposed to antibody while still intact probably means that the epitopes recognized by anti-ECMr on these proteins are not accessible to antibody while the cells are intact.

Immunoprecipitation of blastocysts that were ^{125}I surface-labelled at times during the first 24 hours post-hatching showed no resolvable bands in the 120-160 kD area of the gel, suggesting that these ECMr antigens are either not expressed on the cell surface or are not detectable by my labelling procedures on these earlier stages. These findings correlate with the observation that embryos at this stage are not adherent and do not spread on the substrate during the first 24 hours post-hatching.

Indirect immunofluorescence experiments using anti-ECMr show that ECMr antigens are present on both the ICM and trophoblast outgrowth portions of attached embryos. Observation of the flattened trophoblast cells shows that, although there is quite a bit of diffuse fluorescence, the antigen is enriched on cell processes and on the ventral surfaces of cells. In regions where the trophoblast has been ripped away, there is a characteristic pattern of substrate attached "footpads" like that seen in fibroblastic cells stained with CSAT monoclonal antibody (Damsky, et al., 1985a).

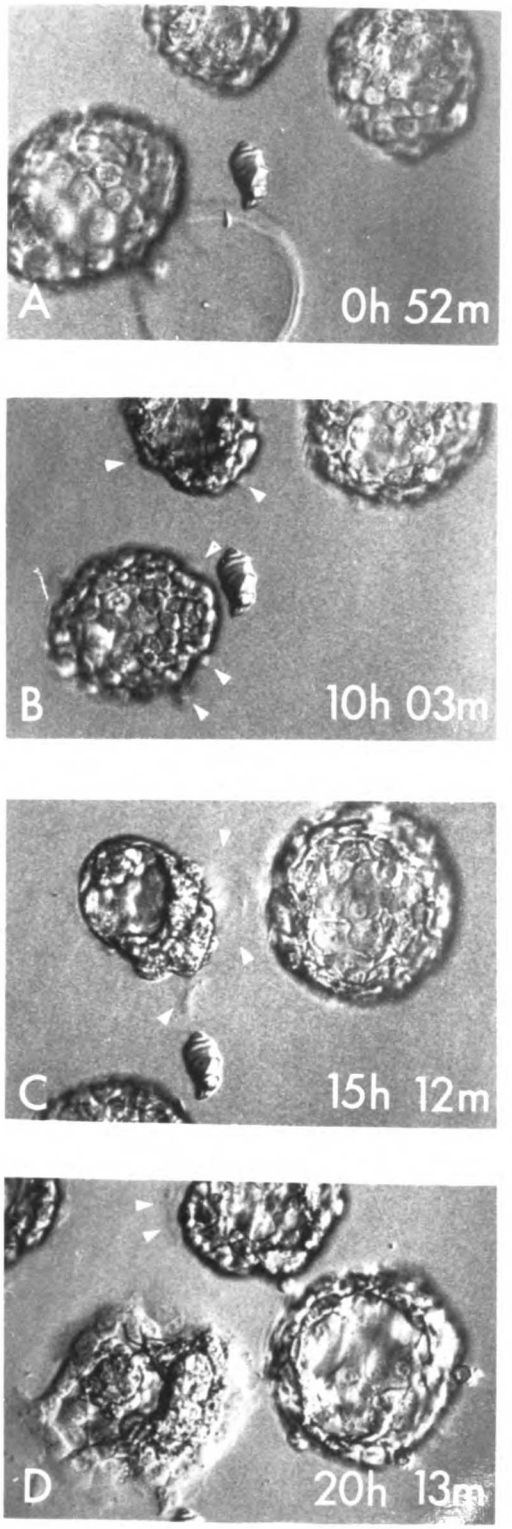
The distribution in trophoblast cells of the adhesion plaque protein Vnc was also examined. This 130 kD intracellular protein has been shown to be a component of adhesion plaques (Geiger et al., 1980; Burridge and Feramisco, 1980). It codistributes with and binds to talin and is proposed to interact indirectly with actin microfilaments (reviewed in Burridge, 1986). In the trophoblast outgrowth of attached blastocysts, anti-Vnc is highly localized in streaks and patches very similar to those found in other

cell types (Geiger, 1979; Burridge and Feramisco, 1980; Geiger et al., 1980; Damsky et al., 1985a). Likewise, the staining pattern seen for talin, which is known to bind to integrin (Horwitz et al., 1986) consists of the same kinds of linear patches and dots, especially at the margins of the trophoblast cells. The staining pattern generated by anti-Vnc colocalizes with that generated by anti-FNR (Figures 3-12 and 3-13). These results strongly suggest that the integrin-like adhesion receptors are found in the adhesion plaques of mouse trophoblast cells, and are thus in an appropriate location to participate in trophoblast cell-substratum adhesion.

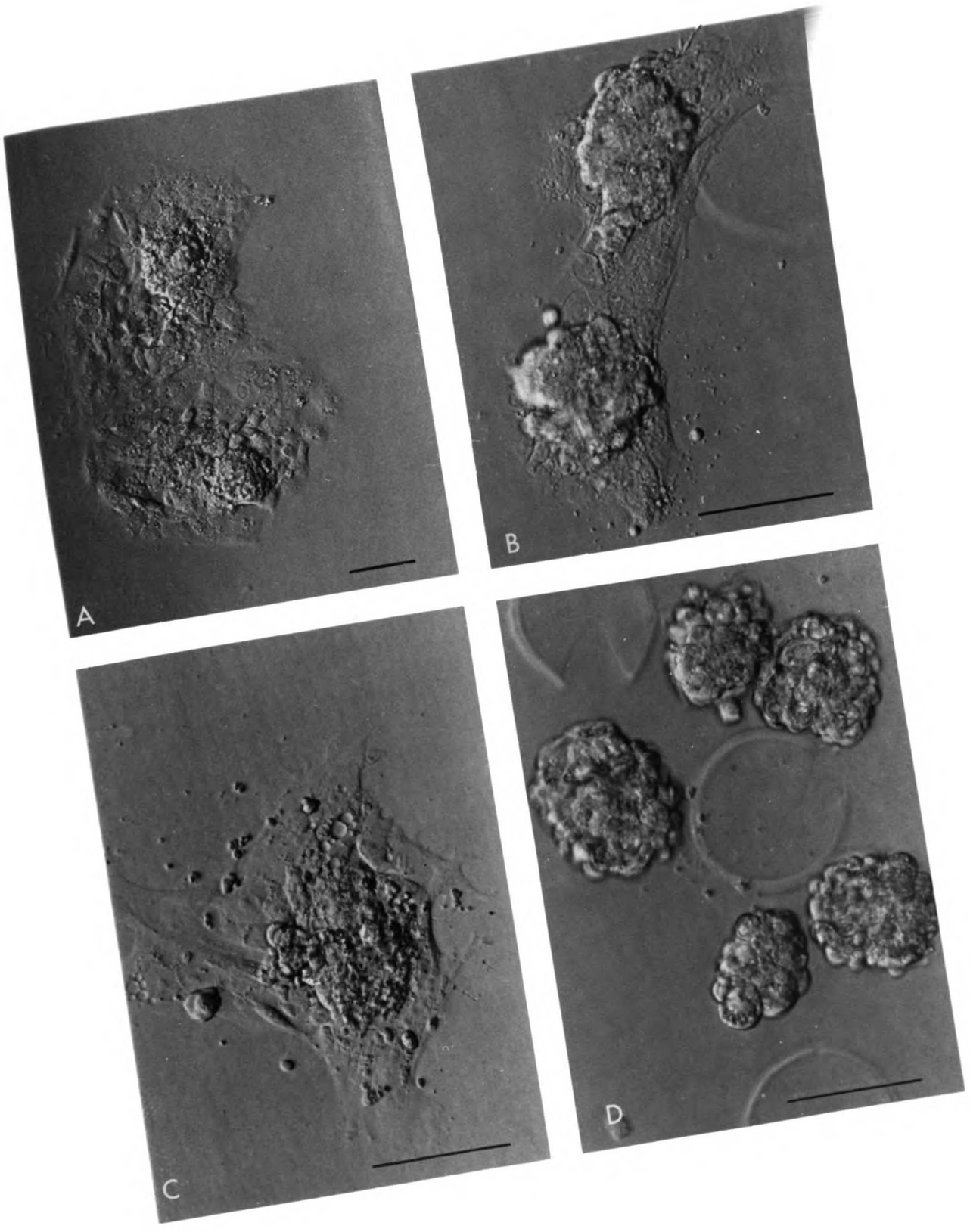
The staining pattern for cytoskeletal and adhesion receptors differs markedly in different parts of the embryo. In the uppermost region of the inner cell mass, ECMr and FNR are distributed uniformly but strongly at the cell surface, while Vnc and talin are distributed diffusely throughout the cytoplasm. This implies that the cells in this region are not associated with one another via well organized junctional complexes, and that adhesion receptors are not well integrated with the cytoskeleton. There is, however, intense cell surface laminin staining in the inner cell mass, that colocalizes with the adhesion receptor staining (not shown). In the apical regions of cells in the region intermediate to the ICM and the trophoblast, both Vnc staining and FNR or ECMr staining are enriched at the cell surface. This may represent a zone that has plentiful adherens junctions. Vnc is a peripheral cytoplasmic component of these junctions, while adhesion receptors such as avian integrin, have been shown to be present in areas of cell-cell as well as cell-matrix contact in sections of embryonic tissue (Chen et al., 1986; Duband, et al., 1986; Krotoski et al., 1986). In the basal region of cells of this intermediate zone and throughout the flat trophoblast area of the outgrowth, ECMr, FNR, and especially Vnc become localized more discretely. The most intense patches of adhesion receptor staining coincide with areas of closest cell-substratum contact as monitored by Vnc staining.

In summary, this study shows that mouse embryos express components of the integrin family of cell surface ECM receptors, as well as a known cytoskeletal ligand for this receptor family, at the time when they are competent to interact with ECM molecules *in vitro*, and with the uterine stroma during implantation. My studies with defined ECM ligands, cell recognition peptides and anti-ECMr suggest that trophoblast cell outgrowth relies strongly on cell interactions with RGD-containing cell-recognition sites on ECM ligands. My results agree with those in other systems showing that these interactions are involved in cell adhesion and migration during development (Boucaut et al., 1984; Bronner-Fraser 1985, 1986; Duband et al., 1986; Grabel and Watts, 1987). The fact that anti-ECMr inhibits outgrowth on, but not attachment to, the complex bovine corneal endothelial cell-derived ECM (Glass and Damsky, unpublished observations), and that RGD peptides inhibit blastocyst outgrowth on but not attachment to Fn and Ln, suggest that blastocysts have a complex overall strategy for reacting with ECM *in vitro*, and by analogy, with the uterine stroma during implantation.

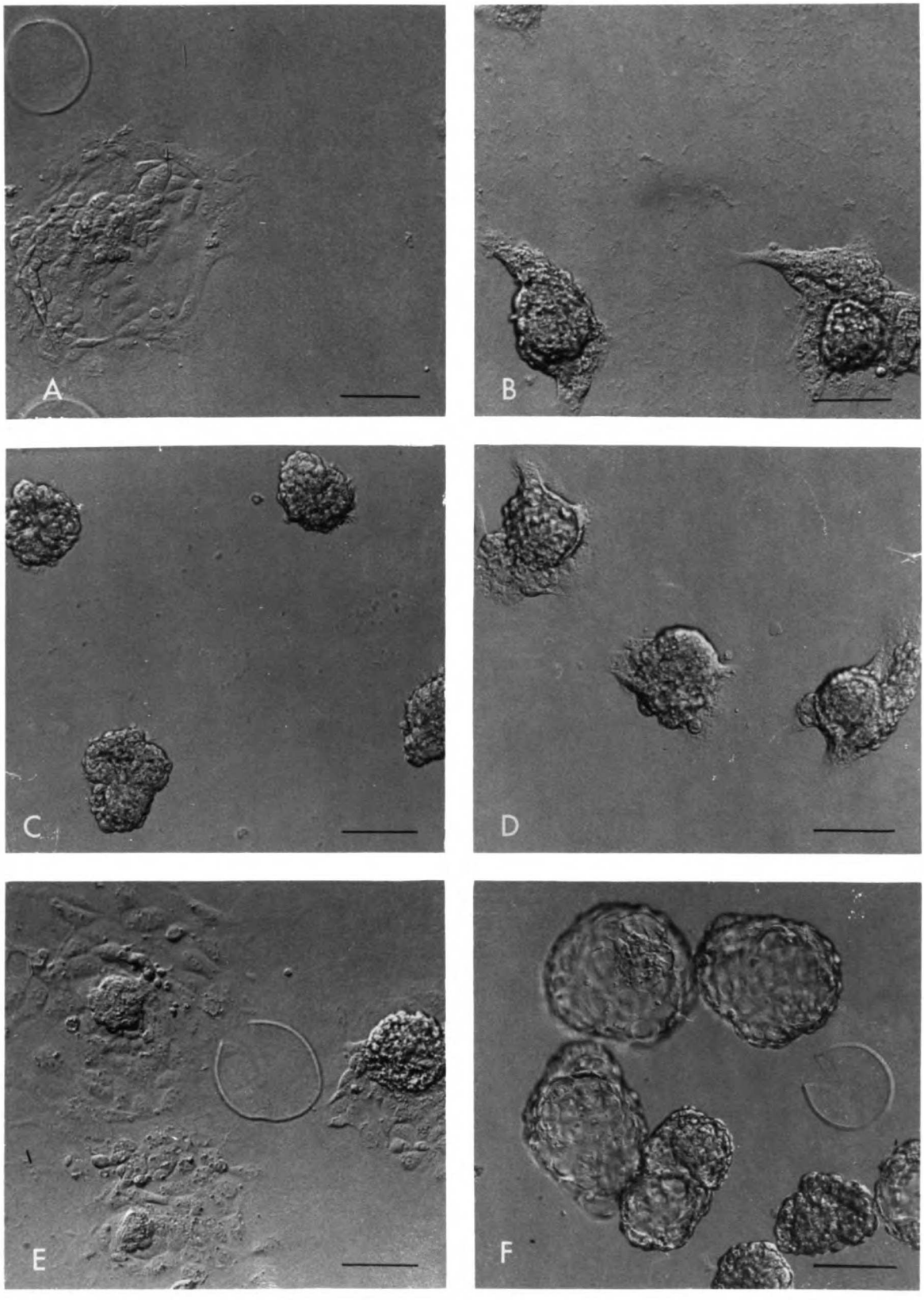
3-1). Cinemicrographic observations of mouse blastocyst outgrowth. Embryos were put into culture for filming at 147 hours post-hCG. A. Immediately post-hatching; none of the embryos are attached, and none show any protrusive activity. B. 10 hours post-hatching; protrusive activity is evident in two of the three embryos (arrows). C. 15 hours post-hatching; spreading is seen in one of the embryos (arrows). D. 20 hours post-hatching; trophoblast spreading has become prominent in one embryo, is just beginning in another, and has yet to begin in a third.


A
0h 52m
B
10h 03m
C
15h 12m
D
20h 13m

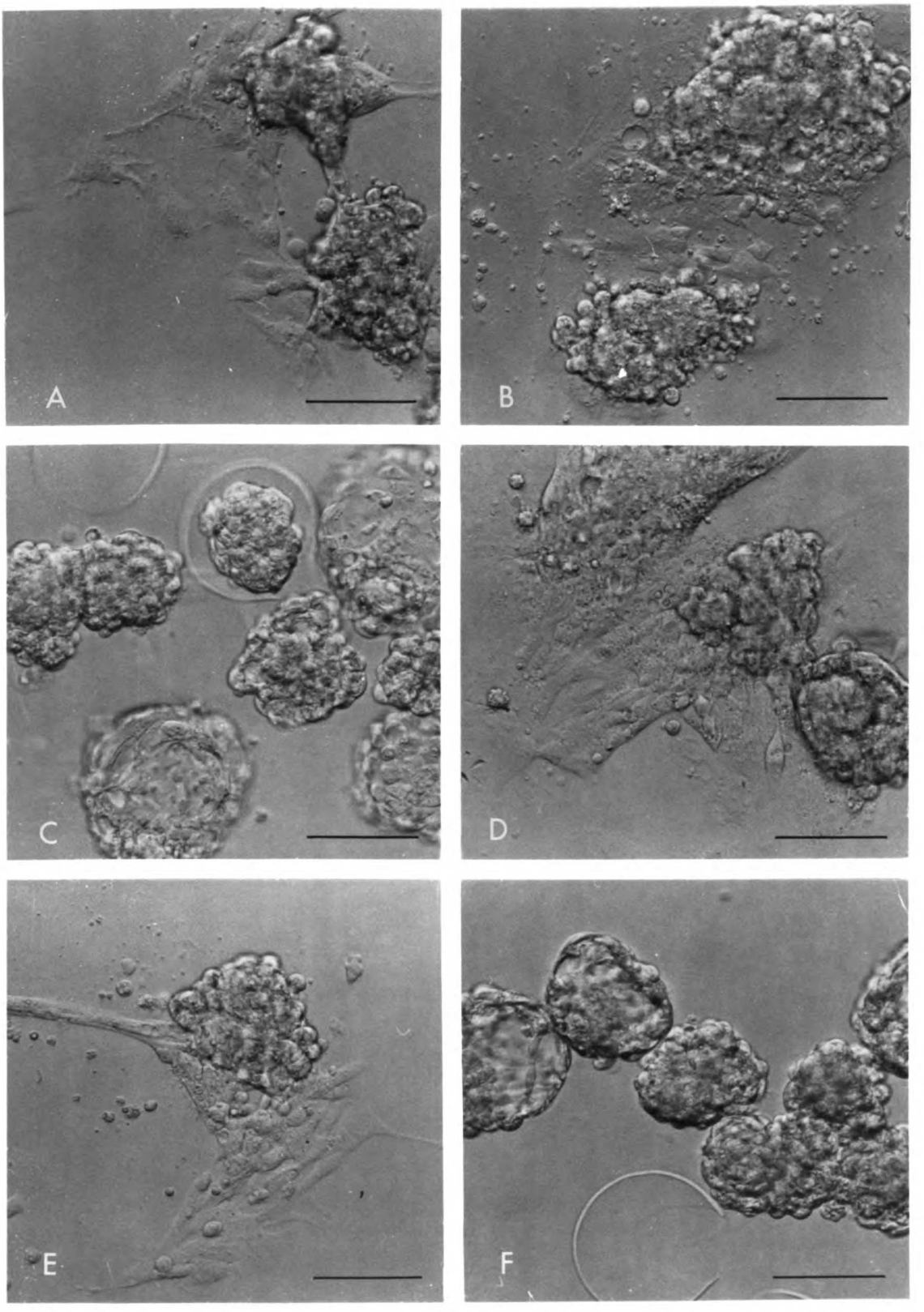
3-2.) Embryo outgrowth on defined substrates in serum-free medium. A. Fn substrate (10 μg/ml). B. Ln substrate (25 μg/ml). C. Col IV substrate (25 μg/ml). D. BSA substrate (25 μg/ml). Scale bars = 100 μm.


A
B
C
D

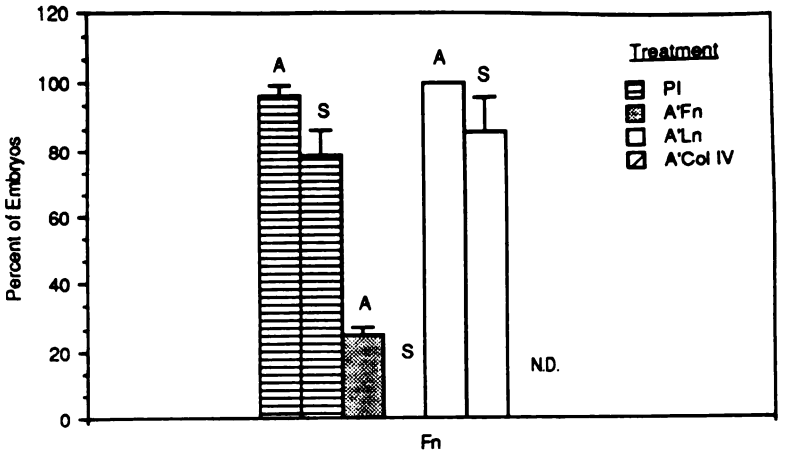
3-3.) Blastocyst outgrowth on either Fn (10 μg/ml) and Ln (25 μg/ml) substrates in the presence of substrate-specific antibodies. A,C,E; Fn substrate. B,D,F; Ln substrate. Embryos were cultured in the presence of: pre-immune rabbit antibodies (A,B); anti-Fn antibodies (C,D); or anti-Ln antibodies (E,F). Scale bars = 100 μm.

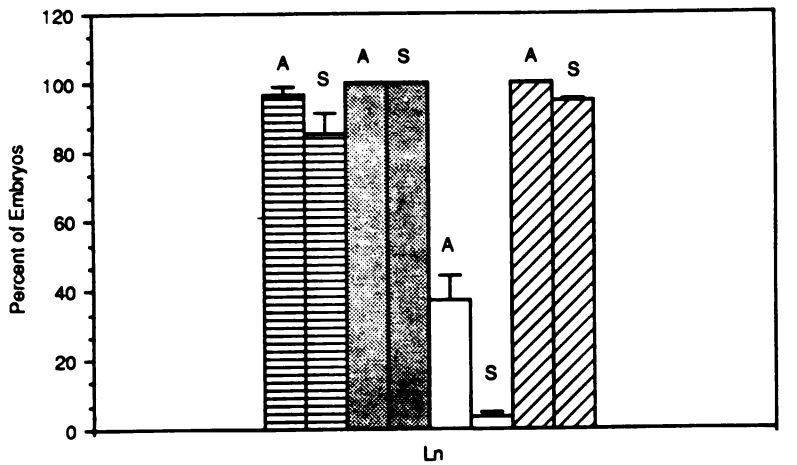

A
B
C
D
E
F

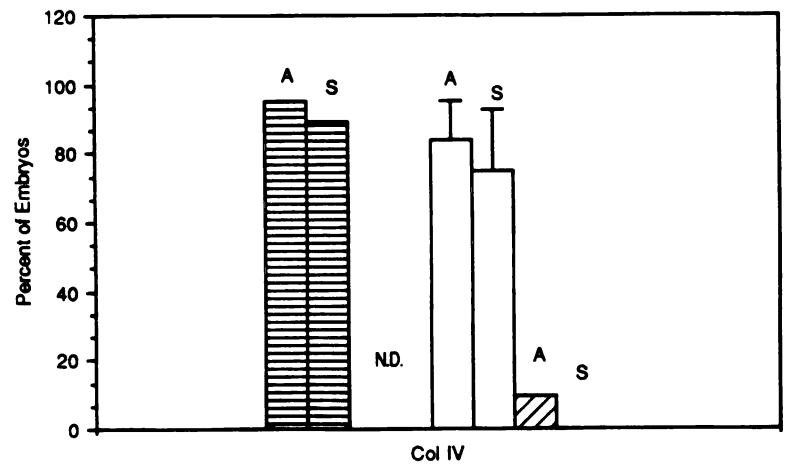
3-4.) Blastocyst outgrowth on Ln (25 μg/ml) and Col IV (25 μg/ml) in the presence of substrate-specific antibodies. A,C,E; Ln substrate. B,D,F; Col IV substrate. Embryos were cultured in the presence of: preimmune rabbit antibodies (A,B); anti-Ln antibodies (C,D); or affinity purified anti-Col IV antibodies (E,F). Scale bars = 100μm.


A
B
C
D
E
F

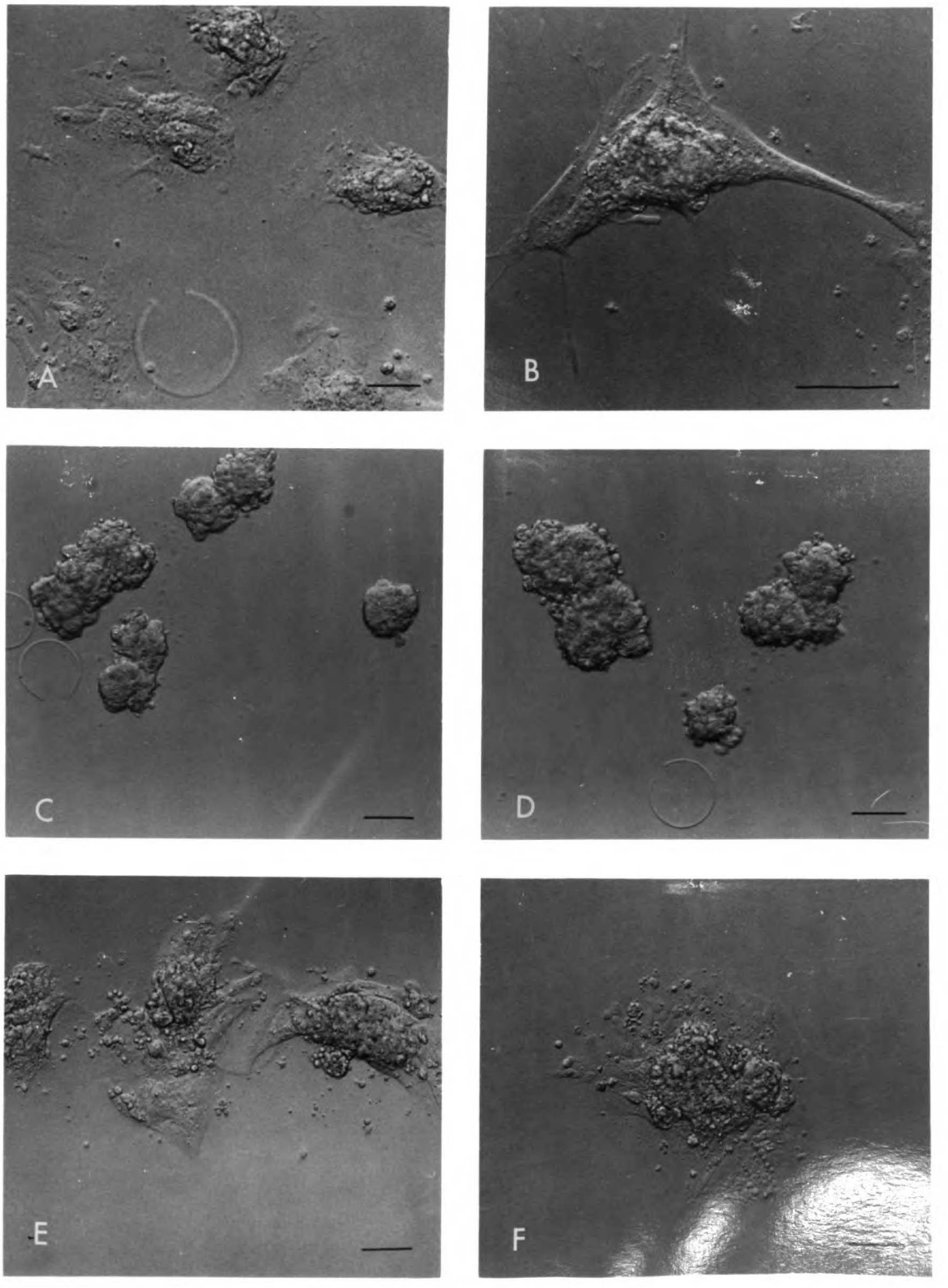
3-5.) Graph of attachment and spreading of mouse blastocysts on Fn (10 μg/ml), Ln (25 μg/ml), and Col IV (25 μg/ml) in the presence of the different substrate-specific antibodies. Bars labelled "A" represent the percentage of embryos that attached to the substrate in each case, while bars labelled "S" represent the percentage that spread on the substrate. N.D.: not done.


Treatment
PI
A'Fn
A'Ln
A'Col IV
Percent of Embryos
120
100
80
60
40
20
0
A
S
A
S
A
S
N.D.
Fn

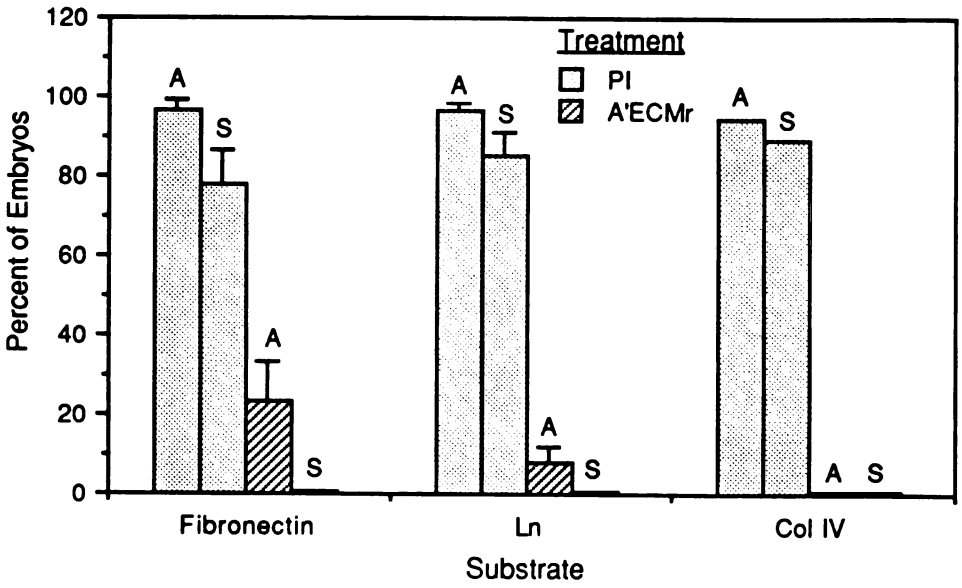

Percent of Embryos
120
100
80
60
40
20
0
A
S
A
S
A
S
A
S
Ln


Percent of Embryos
120
100
80
60
40
20
0
A
S
N.D.
A
S
A
S
Col IV

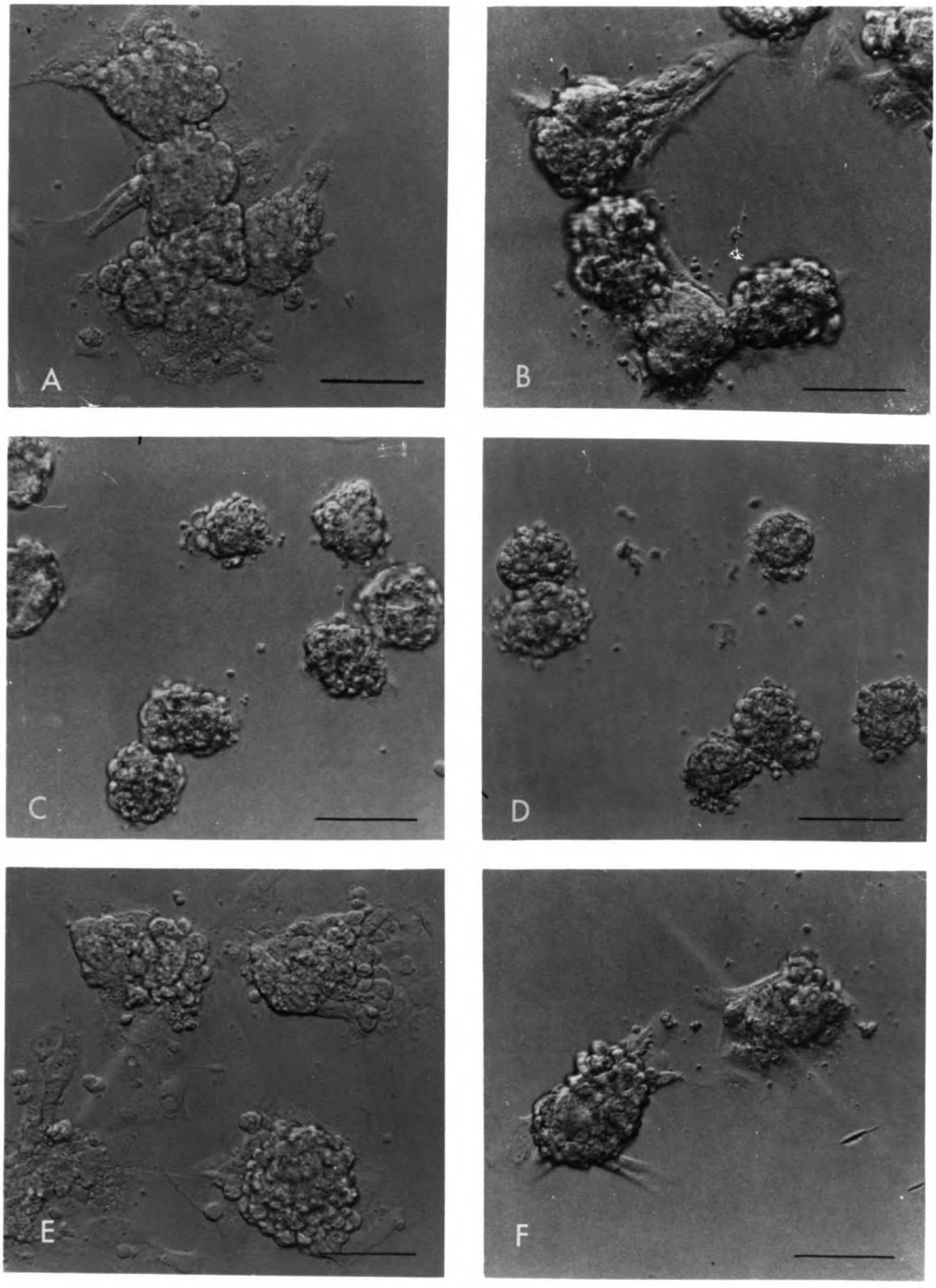
3-6.) Blastocyst outgrowth on Fn (10 μg/ml) and Ln (25 μg/ml) in the presence of anti-ECMr antibodies. A,C,E; Fn substrate. B,D,F; Ln substrate. Embryos were cultured in the presence of: preimmune goat antibodies (A,B) or anti-ECMr antibodies (C,D). E,F: embryos were cultured 48 hours in the presence of anti-ECMr antibodies, then removed, rinsed, and cultured for a further 12 hours in control medium. Scale bars = 100 μm.


A
B
C
D
E
F

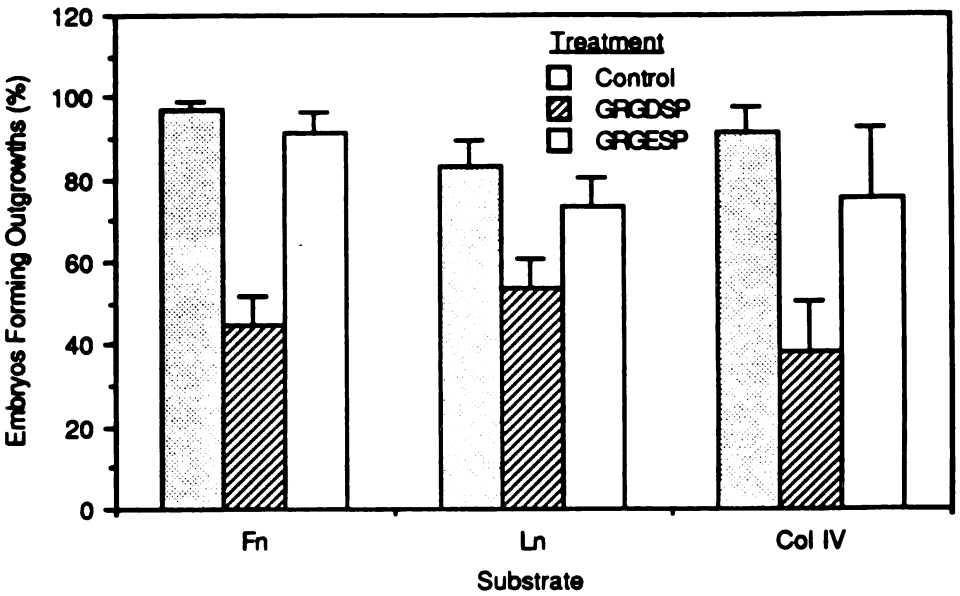
3-7.) Graph of embryo outgrowth on Fn (10 µg/ml), Ln (25 µg/ml), and Col IV (25 µg/ml) in the presence of either preimmune antibodies (PI) or anti-ECMr antibodies (A'ECMr). Bars labelled "A" represent the percentage of embryos that attached to the substrate in each case, while bars labelled "S" represent the percentage that spread on the substrate.


120
100
80
60
40
20
0
Percent of Embryos
Treatment
PI
A'ECMr
A
S
A
S
A
S
A
S
A
S
A
S
Fibronectin
Ln
Col IV
Substrate

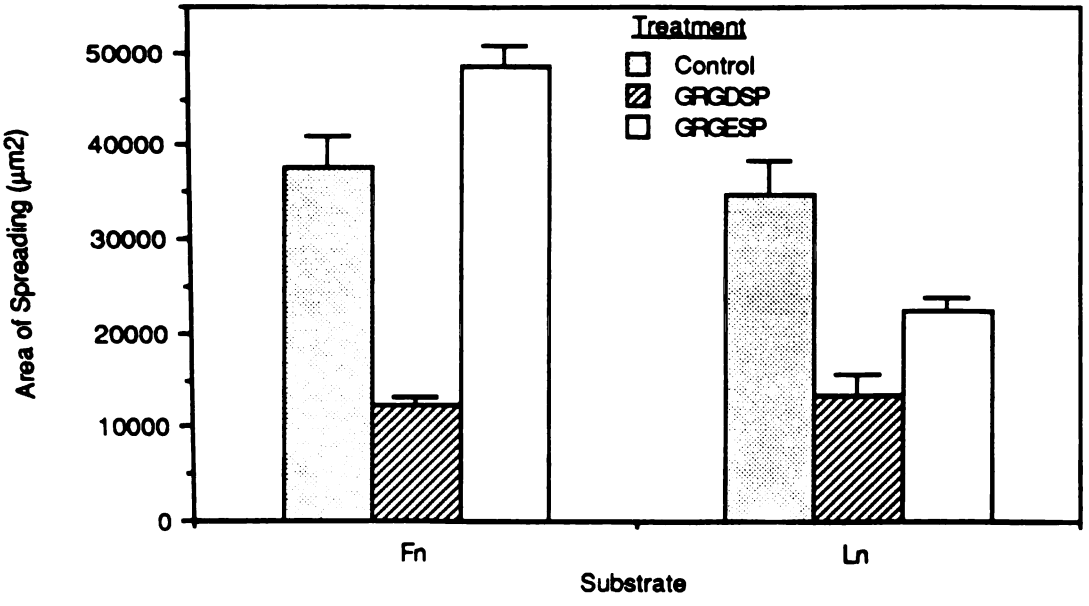
3-8.) Blastocyst outgrowth on Fn (10 μg/ml) and Ln (25 μg/ml) in the presence of GRGDSP and GRGESP synthetic hexapeptides. A,C,E; Fn substrate. B,D,F; Ln substrate. Embryos were cultured in: control medium (A,B); medium containing 500 μg/ml GRGDSP (C,D); or medium containing 500 μg/ml GRGESP (E,F). Scale bars = 100 μm.


A
B
C
D
E
F

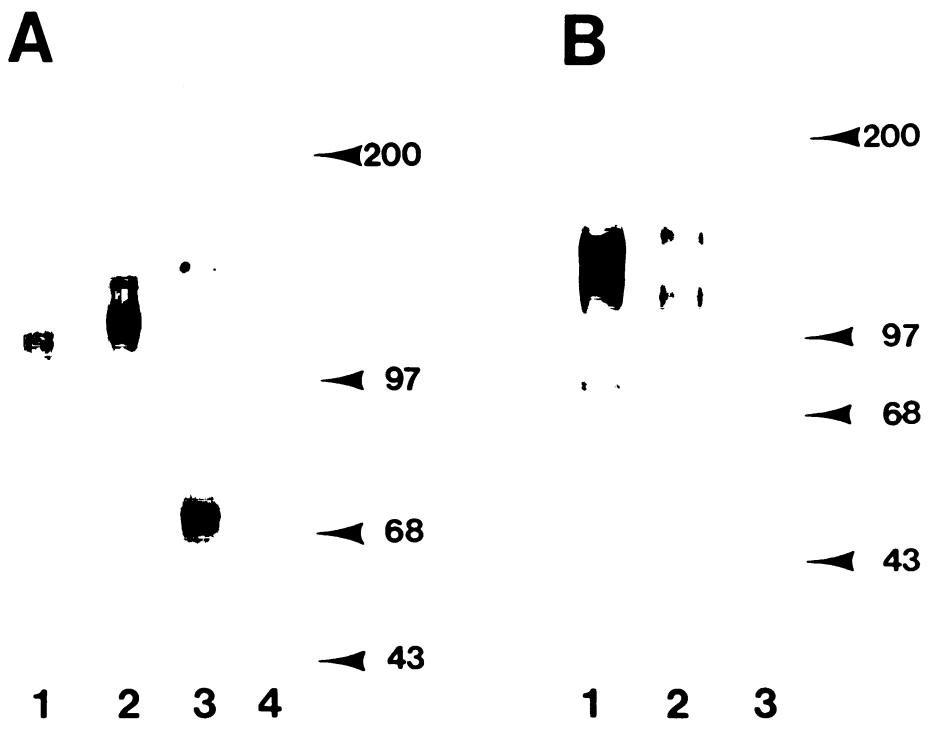
3-9.) Graph showing the percent of embryos which spread on either Fn (10 μg/ml), Ln (25 μg/ml), or Col IV (25 μg/ml) in normal medium (Control), medium containing 500 μg/ml of the active peptide (GRGDSP) or medium containing 500 μg/ml of the control peptide (GRGESP).


Embryos Forming Outgrowths (%)
120
100
80
60
40
20
0
Treatment
Control
GRGDSP
GRGESP
Fn
Ln
Col IV
Substrate

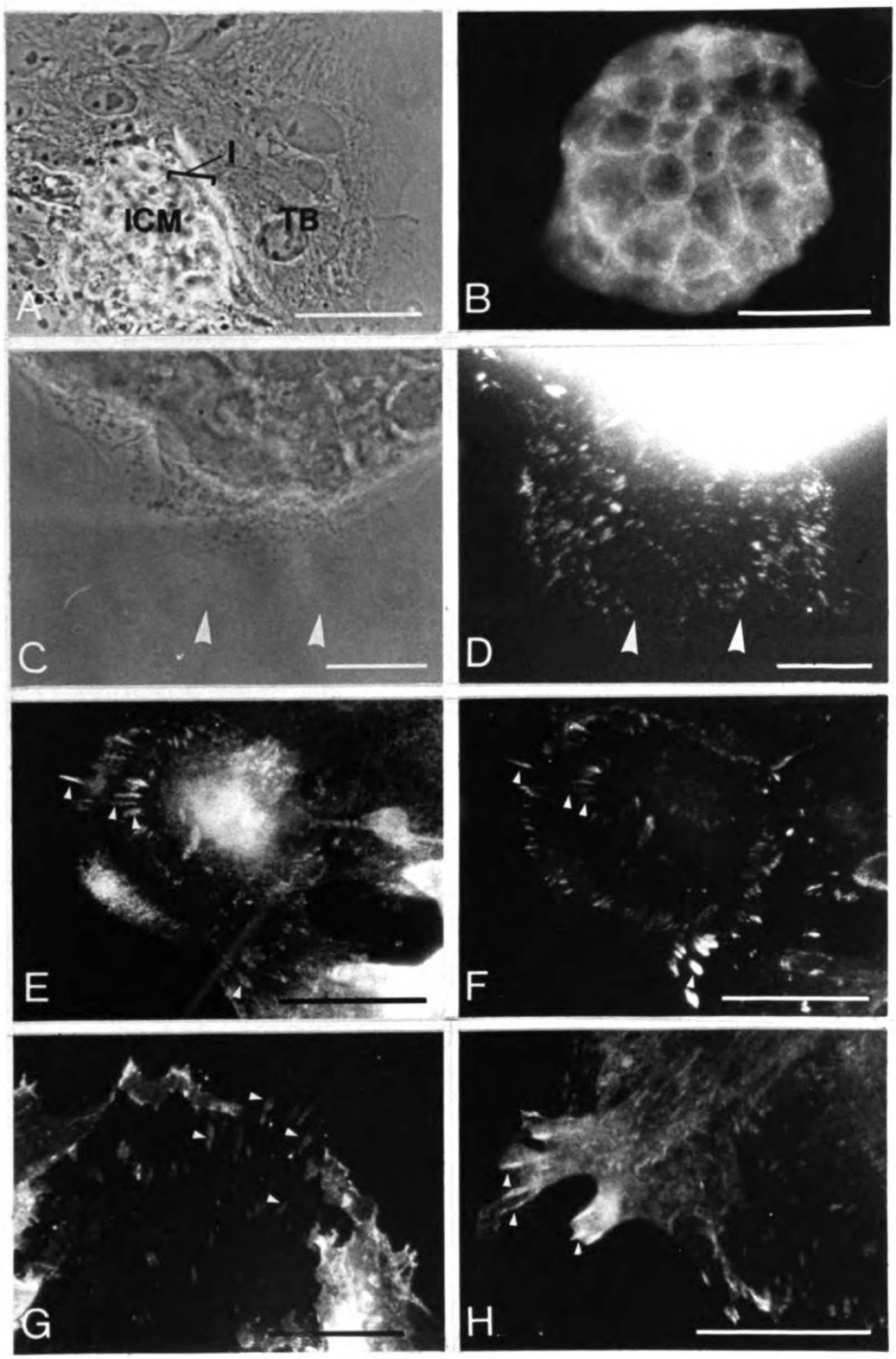
3-10.) Graph showing the area of outgrowth (in μm^2) of embryos cultured on either Fn (10 μg/ml) or Ln (25 μg/ml) substrates in normal medium (Control), medium containing 500 μg/ml active peptide (GRGDSP), or medium containing 500 μg/ml control peptide (GRGESP). Each value represents the mean ± SEM for at least 11 embryos.


Treatment
Control
GRGDSP
GRGESP
50000
40000
30000
20000
10000
0
Area of Spreading (µm2)
Fn
Ln
Substrate

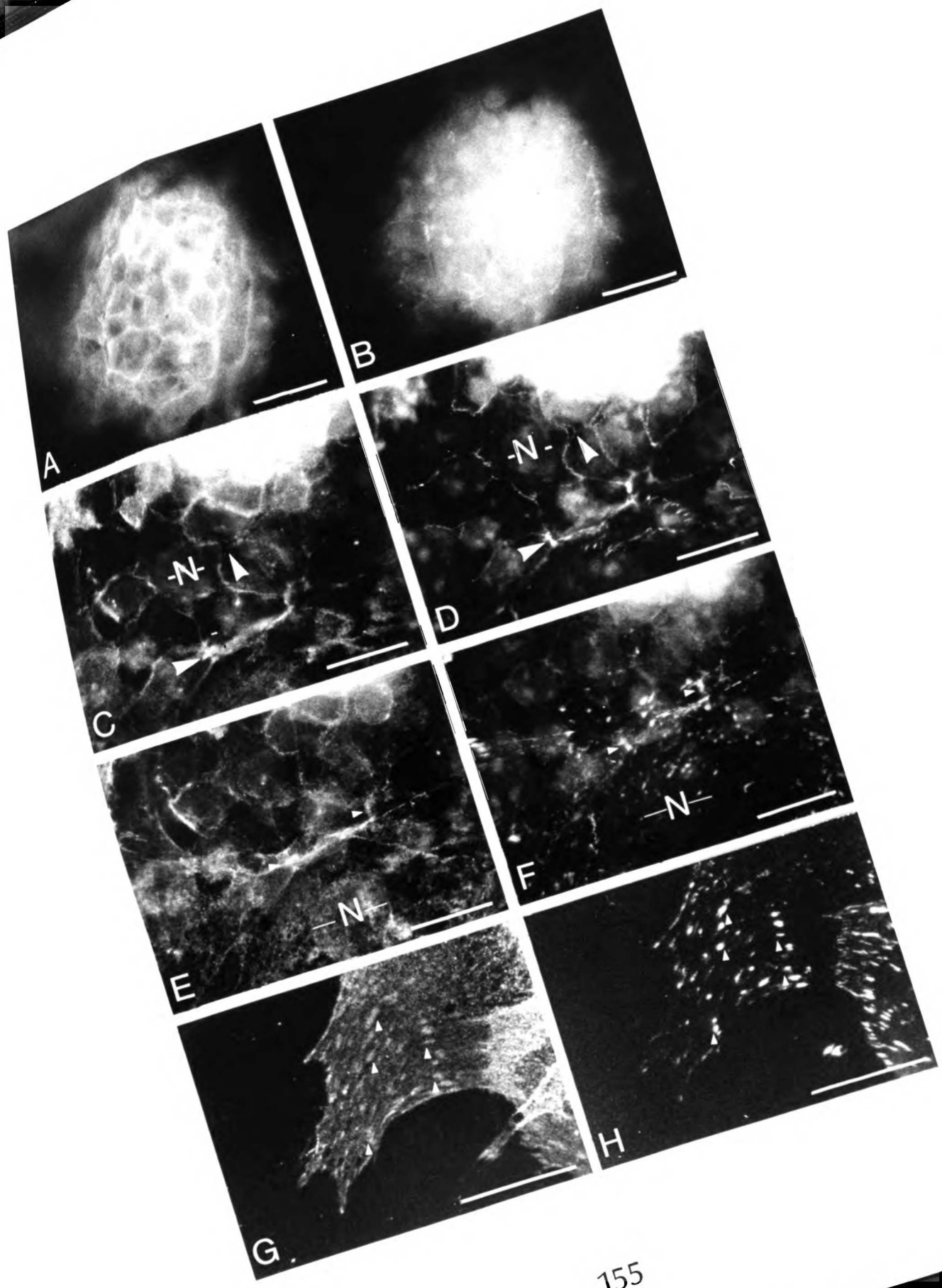
3-11.) Immunoprecipitation of anti-ECMr antigens from cells and embryos. A. Immunoprecipitation by anti-ECMr of ^{125}I-labelled cell surface proteins from 72-hour mouse embryo outgrowths (lane 1), MMTE cells (lane 2), and mouse hatched blastocysts (lane 3). Lane 4; immunoprecipitation by normal goat serum of ^{125}I-labelled cells surface proteins from MMTE cells. Cells and embryos were surface-labelled with ^{125}I, and then incubated in medium containing either anti-ECMr or preimmune antibodies prior to lysis (see Materials and Methods). B. Immunoprecipitation of ^{125}I-labelled cell surface proteins from 72-hour mouse embryo outgrowths (lane 1) and and JAR cells (lane 2). Lane 3; immunoprecipitation by normal goat serum of ^{125}I-labelled cell surface proteins from JAR cells. Cells and embryos were surface-labelled with ^{125}I, then lysed and the labelled lysate subjected to immunoprecipitation with either anti-ECMr or preimmune antibodies. Immunoprecipitation by normal goat serum of the above embryo samples were similar to those shown for MMTE cells.


A
200
97
68
43
1 2 3 4
B
200
97
68
43
1 2 3

3-12.) Immunofluorescent staining of antigens recognized by anti-ECMr, anti-FNR, anti-Vnc, and anti-talin antibodies on blastocyst outgrowths. A. Phase photograph of an outgrowth, demonstrating the regions shown in the other photographs. ICM - inner cell mass. TB - trophoblast cells. I - intermediate region. B. Anti-ECMr staining is concentrated at the cell surface in the ICM, outlining the cells. C. Phase photograph of trophoblast cells beginning to spread in a 48-hour outgrowth. D. Anti-Vnc staining in the same early outgrowth, demonstrating organization of cell-matrix contacts even in early stages of outgrowth. E and F. Anti-FNR (E) and anti-Vnc (F) double staining on a 48-hour outgrowth. Adhesion receptors and Vnc colocalize in discrete cell-matrix contact sites (arrowheads). G. In areas where the trophoblast has been torn away from the substrate, anti-ECMr staining is seen on the substrate in bright strips and dots (arrowheads). H. Anti-talin fluorescence is seen in similar patterns in peripheral processes of trophoblast cells (arrowheads). Scale bars = 25 μm.


I
ICM
TB
A
B
C
D
E
F
G
H

3-13.) Double staining for anti-FNR and anti-Vnc on a 72-hour outgrowth, examined in various regions of the outgrowth. A,C,E,G; anti-FNR staining. B,D,F,H; anti-Vnc staining. A and B. Anti-FNR is localized at the surface of cells in the top of the ICM, while anti-Vnc staining is diffuse and only slightly above background. C and D. When focussing on the apical ends of cells found in the region intermediate to the ICM and the trophoblast, both anti-FNR and anti-Vnc staining are observed to be localized at the cell surface (arrowheads). N - nucleus. E and F. When focussing more basally in the same region pictured in C and D, anti-Vnc staining is seen in a discrete, punctate pattern. Anti-FNR staining is enriched in areas of discrete Vnc staining, but is much more diffuse. Arrowheads point to some areas of codistribution. N - nucleus. G and H. In the highly spread trophoblast cells at the periphery of the outgrowth, both anti-FNR and anti-Vnc staining are organized into a series of discrete cell-matrix contacts. Arrowheads point to some areas of codistribution. Scale bars = 25 μm.


A
B
C
D
E
F
G
H
N
N
N
N

Summary

The results described in this thesis address three main issues in the early development of the mouse embryo. Chapter One describes a cinemicrographic analysis of early cleavage stages, and provides new information on the process of allocation of 16-cell blastomeres to either the trophectoderm or inner cell mass lineages. Chapter Two describes an scanning electron microscope analysis of compaction and polarization using cytoskeletal inhibitors to determine the mechanisms responsible for these two processes as well as to describe their morphology. Chapter Three is a study on the formation of outgrowths of mouse blastocysts on substrates of extracellular matrix molecules and on the role of the integrin family of cell surface extracellular matrix receptors in this process.

Compaction is a slow and variable process that can extend over more than one cell cycle, and comprises two components, cell flattening and polarization. Since polarization of the blastomeres is an important preliminary to the segregation of the inner and outer cells of the morula beginning during fourth cleavage, it is important to understand the factors controlling compaction. My results show that microfilaments are responsible both for cell flattening and for the polar redistribution of surface microvilli, whereas microtubules are not involved in either. While the mechanics of cell flattening and polarization have been well researched, many important aspects of the compaction process remain to be resolved. For example, it is still not clear to what extent cell flattening and polarization are linked processes. More importantly, the factors governing the onset and rate of both cell flattening and polarization are still unknown.

The foundation of two populations of blastomeres in the mouse morula begins during the transition from the 8- to the 16-cell stage, when some of the blastomeres become completely enclosed within the embryo. It has long been assumed that the allocation of cells to the inner cell population was the result of a delaminatory division of 8-cell blastomeres. My results show that the orientations of cleavage planes in the intact 8-cell embryo are more variable than previously thought, and include oblique division planes. The results presented are consistent with the notion that the orientation of division planes during fourth cleavage determines the positional fate of the daughter blastomeres in the morula, however, a direct correlation remains to be shown. The preponderance of evidence supports the idea that the apical region of the 8-cell blastomere constrains the fate of the daughter cell(s) to which it is allocated, but the extent and nature of its influence is unclear. Whether the observed constraint is dependant on internal or external constituents also remains to be resolved. There is no association between division order at either the 2-, 4-, or 8-cell stages and division plane orientation during fourth cleavage, even though division order has been correlated with extent of allocation of descendants to the inner cell population. To resolve this paradox, an examination of the correlation between phenotype and position of the daughter cells of oblique divisions becomes critical. Such an examination would also shed light on the nature of the putative apical restraining factor as well as the extent to which mechanical forces within the 8-cell embryo influence allocation.

Following hatching from the zona pellucida, the trophectoderm of the mouse blastocyst differentiates to form trophoblast. The primary function of these cells is attachment to the uterine lining and invasion of the uterine stroma. Interactions between the trophoblast cells and the surrounding extracellular matrix are thought to be an important part of this process. In culture, mouse embryos will specifically recognize and form outgrowths on substrates of defined extracellular matrix molecules.

The results described in Chapter Three show that components of the integrin family of extracellular matrix receptors begin to be expressed in mouse embryos only some time after hatching, and are involved in attachment and outgrowth on substrates of fibronectin, laminin, and collagen type IV. The data also suggest that reaction with these substrates is complex, and involves more than one type of adhesive interaction. The role that these extracellular matrix receptors play in implantation in the uterus needs to be examined, as does the relationship between the receptors found in the embryo and those characterized in other cells.

In conclusion, the experiments described in this thesis address several aspects of the formation and function of trophectoderm during the early development of the mouse embryo. The results show that the compaction blastomeres at the 8-cell stage is a gradual process involving flattening and polarization of the blastomeres, that is effected by microfilaments. The orientation of the division planes during the subsequent cleavage division then provides the basis for the segregation of the two populations of blastomeres in the morula. The outer cells will give rise to the trophectoderm of the blastocyst, which then differentiates to form trophoblast after hatching of the embryo from the zona pellucida, while the inner cells give rise to the inner-cell mass, and ultimately, the embryo proper. During implantation, trophoblast cells interact with the extracellular matrix of the uterine stroma, and this is reflected in their ability to form outgrowths on defined substrates of extracellular matrix molecules.

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