# Segregation of cell surface antigens during early development of mouse gives positional information for blastocyst differentiation

MARIA INES BECKER[1,2], ALFREDO E DE IOANNES[2]
and the late LUIS IZQUIERDO[3]

[1]Laboratorio de Inmunología, Bios Chile Ingeniería Genética, Santiago, Chile.
[2]Unidad de Inmunología, Facultad de Ciencias Biológicas,
Pontificia Universidad Católica de Chile, Santiago, Chile.
[3]Laboratorio de Biología del Desarrollo,
Facultad de Ciencias, Universidad de Chile, Santiago, Chile.

*We studied the reactivity of 22 rat monoclonal antibodies directed against cell surface antigens shared by mouse preimplantation embryos and F9-teratocarcinoma cells, using indirect immunofluorescence. Monoclonal antibodies were classified in 8 categories, according to the localization of the antigen in the oocytes and in the cell mass or trophoblast from in vitro implanted blastocysts. This antigen distribution reveals that we obtained lineage markers for trophoblastic cells and for cell mass. The existence of cell surface antigens present since oogenesis and localized thereafter only in the trophoblast, and on the other hand, the absence of cell surface antigens present since segmentation and thereafter localized exclusively in the trophoblast, led us to propose a model in which the trophoblast determination would start during oogenesis and would occur by the segregation of components towards the periphery of the embryo.*

**Key words:** *cell surface antigens, monoclonal antibodies, mouse development.*

## INTRODUCTION

The process of cell differentiation and morphogenesis occurring in early mammal development culminates with blastocyst formation, in which the two first tissues may be distinguished: the inner cell mass which gives rise to the embryo, and the trophoblast which will originate part of the embryonic annexes. In this early period of development, the issue arises of how to explain the cell differentiation observed in the blastocyst, from a fertilized egg in which no cytoplasmic regions may be distinguished and whose segmentation generates cells constituted by similar cytoplasm, which surround apparently identical nuclei.

Early reaggregation studies of embryo cells in sea urchin (Giudice, 1962; McClay and Chamber, 1978), sponges (Moscona, 1968), fungi (Beug *et al*, 1970, 1973) and chicken neural cells (Thiery *et al*, 1977; Rutishauser and Edelman, 1978), showed that embryo cells may be specifically recognized for histotype and functional complementarity. The molecules involved in this phenomenon can be neutralized by antibodies. This indicates that embryo cells from different tissues have cell surface antigens, which function as intercellular signals or ligands (Goldschneider and Moscona, 1972; Babinet and Condamine, 1980). In this context, cell differentiation would be reflected in the appearance of a

**Correspondence to:** María Inés Becker, Bios Chile I.G.S.A., Av. Marathon 1493, Santiago, Chile. Fax: (56-2) 239-4250.

single qualitative, quantitative and structural molecular pattern on the cell surface (Monod and Jacob, 1961).

It has been postulated that in mammals the embryonic cell surface molecules would play an important role in the regulation of cell interactions and cell sorting during early development and differentiation, however, no cell surface antigens have been described to be exclusive to some stage of preimplantation development, allowing to establish in the morula the inner cell mass-trophoblast lineage. The available literature shows that molecules that could be characteristic, but not exclusive, of embryonal cells are expressed during this period. This would be the case of antigens expressed in tumoral cells such as teratocarcinomas. These cells share some properties with the embryos and can replace them in immunological and biochemical manipulations (Artzt *et al*, 1973; Graham, 1977; Martin, 1980).

Two-cell mouse embryos cultured in the presence of antiserum to F9-teratocarcinoma cells (TC-F9) develop normally, however, when cultured in the presence of Fab fragments of the antibodies, no compaction or blastocyst formation is observed (Kemler *et al*, 1977, 1979). Johnson and Calarco (1980a), using anti-blastocyst serum, showed the presence of a group of glycoproteins, of 65 and 70 kDa, whose expression began during segmentation and was maximal at the 8-cell stage. Furthermore, the neutralization of these molecules by the same antibodies arrested development. These authors suggested that such molecules correspond to antigens that are expressed only at this developmental stage. However, the use of antiserum hinders the interpretation of the results, since cross reactions persist even after exhaustive adsorptions to increase specificity, and precludes a precise definition of embryo stage-specific antigens. On the other hand, the existence of molecules exclusive to this period could correspond to molecules encoded by the mouse T locus. In fact, t12/t12 embryos are not compacted and stop their development (Bennett, 1975; Silver, 1986).

Using monoclonal antibodies against embryonal cells, differentiated tissues and tumoral cells, several cell surface antigens of the preimplantation embryos have been defined as differentiation antigens (Johnson and Calarco, 1980b). Nonetheless, this term is ambiguous. We think that it requires a more precise definition, since it includes both the stage-specific and tissue-specific antigens. As shown in Figure 1, an antigen recognized during morphogenesis or cell differentiation, but that is not recognized thereafter in terminally differentiated cells, is actually a stage-specific antigen; while an antigen recognized in a differentiated cell type and serving as marker to distinguish it from another one, is actually a tissue-specific antigen. The significance of these different categories for the analysis of development must not be misunderstood; in fact, a stage-specific antigen may be at the same time a

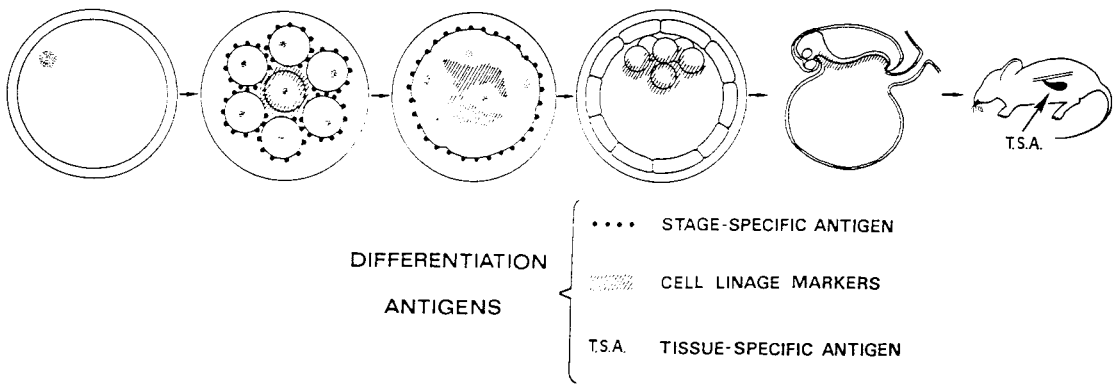


Fig 1: Definition of cell surface antigens considered as differentiation antigens. *Stage-specific antigens*, molecules expressed at a precise stage of development in which they play an important role; they are not expressed thereafter. *Cell-lineage markers*, molecules expressed in an embryonic tissue, serving as markers to distinguish it from other embryonic tissues. *Tissue-specific antigens*, molecules expressed by terminally differentiated tissues.

tissue-specific antigen. If we also consider as tissue-specific those antigens that can be recognized in cell types that have not reached their terminal differentiation, there would be an intermediate category corresponding to the so-called cell lineage markers.

In the present work, we used monoclonal antibodies to mouse morulae and TC-F9 cells to seek stage-specific antigens and cell lineage markers, allowing the lineage cell mass-trophoblast in the morula to be traced.

## MATERIALS AND METHODS

### *Biological material*

*Oocyte and follicular cells.* CF1 mature females were sacrificed by cervical dislocation; the ovaries were isolated in phosphate buffer saline (PBS). They were then ground with a metallic spatule and cell debris was discarded. The oocytes were collected with a fine pipet and placed into a capsule containing Biggers medium (Biggers *et al*, 1971). In this study, we only considered oocytes of normal appearance, *i.e.*, transparent cytoplasm, visible germ cell, lack of perivitelline space and complete zona pellucida. In some oocytes, follicular cells were not eliminated.

*Mouse preimplantation embryos.* CF1 female mouse of 6 to 8 weeks old were induced to superovulate with an intraperitoneal injection of 5 IU of pregnant mare gonadotrophin (Sigma) followed 44 h later by an intraperitoneal injection of 5 IU of human chorionic gonadotrophin (Sigma). After mating, the females were sacrificed by cervical dislocation and the embryos were obtained by perfusing the oviduct or the uterus with Biggers medium supplemented with 4 mg bovine serum albumin (BSA)/ml. Embryonic development was counted from time 0 of the day in which the vaginal plug was detected. Two-cell embryos, morulae and blastocysts were collected at approximately 36, 60 and 85 hours of development, respectively.

*Implanted blastocysts.* The procedure described by Fernández and Izquierdo (1980) was used. In brief, day 4 blastocysts were recovered by perfusing the uterus with medium supplemented with BSA (4 mg/ml) (Biggers *et al*, 1971). The blastocysts were washed with NCTC-135 medium (GIBCO) and distributed in 96-well plates (NUNC) or in culture capsules (Falcon) containing the following implantation medium: NCTC-135 supplemented with 10% bovine fetal serum, 100 IU penicillin and 100 IU streptomycin. Embryos were incubated for 4 days at 37° C, in a humid atmosphere with 10% $CO_2$.

*F9-Teratocarcinoma cells.* They were donated by MS Sherman of the Roche Institute, NJ, USA. The cells were cultured in plastic bottles or in plates previously treated with 1% gelatin. In brief, they were grown routinely at 37° C in a humid atmosphere and 10% $CO_2$ in Dulbecco's medium with 10% bovine fetal serum and antibiotics. To expand cultures, the cells were collected using Trypsin-EDTA. When immunizing with these cells they were collected by washing several times with PBS, then incubated with the same medium used to harvest the cells.

*Spermatozoa.* Mature CF1 male mice were sacrificed as mentioned above and the tail of the epididymis and part of the deferent duct was removed. Both tissues were perfused with medium or PBS (Biggers *et al*, 1971). Smears were then prepared with 15 µl of the sperm suspension, they were left to dry in air and subsequently fixed for a period of 20 min in 3.7% paraformaldehyde in PBS.

*Blood cells.* Blood was collected from the caudal vein; then, smears were prepared and left to dry in air before fixation. Mouse lymphocyte smears were also prepared by blood centrifugation in Ficoll-Hypaque medium.

### *Hybridoma production*

Ten week old Sprague-Dawley rats were immunized with mouse morulae of approximately 60 hours of development. Each rat received 3 intravenous injections of 200 morulae over a 3 month period. Ten days after each immunization the rats were bled

Biol Res 26: 441-452 (1993)

through the tail to obtain the serum for measuring the serum antibody titer by indirect immunofluorescence. At the same time, rats of the same age were immunized with 1 x $10^6$ TC-F9 cells using the intravenous route. The rats with the best antibody titer received an intravenous booster and a somatic fusion of the splenic lymphocytes with cells of the myeloid line NSO/2 was performed, according to the protocol described by Köhler and Milstein (1975). Hybridomas secreting monoclonal antibodies against cell surface antigens of embryos or TC-F9 cells, were selected by indirect immunofluorescence with 8-cell morulae or with TC-F9 cells respectively. The subclass of monoclonal antibodies was determinated with a commercial kit from Zimed Lab.

*Immunocytochemical techniques*

*Indirect immunofluorescence.* Samples were used without fixation, or fixed in a 3.7% paraformaldehyde solution in culture medium or PBS, for 20 to 30 min at room temperature. They were then thoroughly washed with PBS and incubated in 0.2 M glycine for 1 h to block the unreacted paraformaldehyde. Incubation with the first antibody was carried out for 1-2 h at 37° C or overnight at 4° C, with the undiluted hybridomas supernatant. They were then washed three times with PBS for 5 min. For developing, rabbit or goat serum anti-rat immunoglobulin labeled with fluorescein isothiocyanate (Sigma) was used, diluted 1:40 with 1% PBS-BSA. They were washed as above, and then finally washed again with 0.05% PBS-Tween for 3 min to eliminate non-specific binding.

To determine the specificity of the reaction, the following controls were conducted: incubation with rat serum against TC-F9 cells, incubation only with conjugate, and incubation with the supernatant of the hybridomas parental line (NSO/2).

In some cases, a contrast stain containing 0.5% Evans Blue in PBS was used. DABCO solution was used as the mounting medium (Harlow and Lane, 1989). The preparations were observed under a Nikon or Zeiss microscope equipped with epifluorescence.

## RESULTS AND DISCUSSION

*Development of monoclonal antibodies*

Two somatic fusions were performed, one using the spleens of a rat immunized with mouse morulae and the second using TC-F9 cells. According to the primary hybridoma selection, all supernatants presenting some reactivity with TC-F9 cells by indirect immunofluorescence were considered as positive. Figure 2 shows some patterns of antigen localization on such cells, the distribution outlines its polygonal contour with varying intensities of the fluorescence.

Afterwards, using indirect immunofluorescence, we studied the localization of antigens recognized by monoclonal antibodies in oocytes obtained from the ovary, and in tissues of the implanted blastocyst. The cell mass and the trophoblast are exposed in the implanted blastocysts, hence the antibodies freely reach both tissues. From this study we selected 43 antibodies that recognized antigens shared by TC-F9 cells and embryos; however, after expansion only 22 of them kept their properties. This instability could be due to the heterologous nature of the hybridomas.

We classified the antibodies in 8 categories, according to the presence or absence of the antigen (Table I). It is worth noting that this Table includes 2 groups (II and VII) which corresponds to a theoretical category. We have not found in our panel monoclonals that bind to antigens present from the oocyte stage and that are expressed only in the cell mass during implantation (Group II); neither have we found antibodies reactive to antigens which appeared during segmentation and that thereafter were localized only on the trophoblast (Group VII).

Western blot did not allow the further characterization of antigens recognized by the monoclonal antibodies, as the results with this technique were negative, probably because most of them belong to the IgM class, and would also recognize conformational epitopes. The limitations of monoclonal antibodies used with these techniques have been widely documented (Feizi 1985; Anderson *et al*, 1987; Harlow

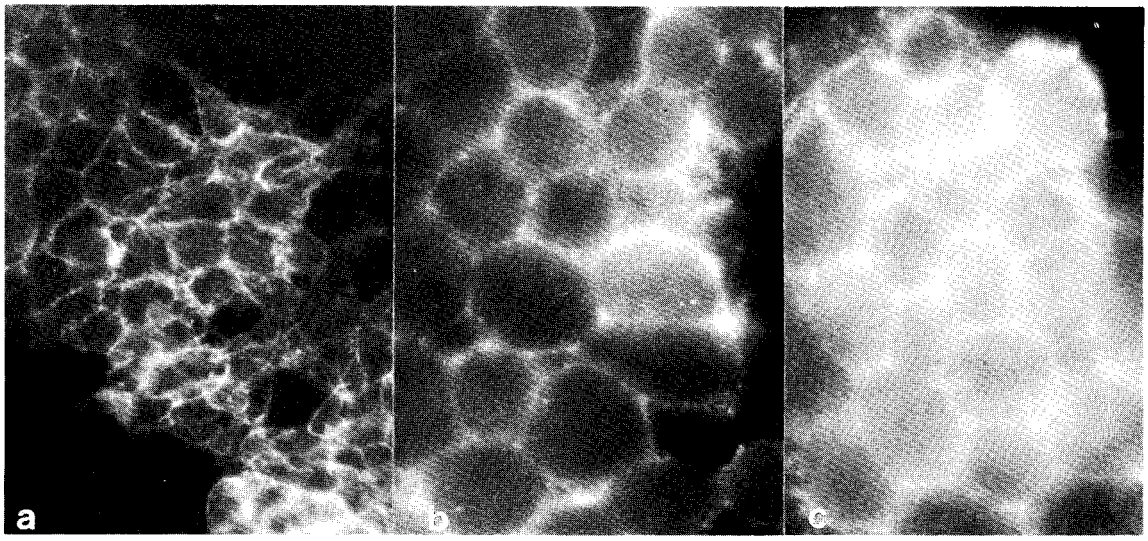


**Fig 2: Patterns of antigen localization recognized by some monoclonal antibodies on TC-F9 cells, using indirect immunofluorescence. a,** Mor-F2A6 monoclonal antibody (Group I). The fluorescence shows the typically polygonal morphology of the TC-F9 cells, x 310; **b,** TC-4E5 monoclonal antibody (Group III), 700 x; **c,** TC-3H5 monoclonal antibody. Note the intense fluorescence on the whole surface of the cells, x 700.

and Lane, 1989). It could be stated that there are 6 cell surface antigens, one for each of the established groups, excepting groups II and VII. However, the localization patterns of the antigens on TC-F9 cells and embryos suggest more than one target molecule per group. Using an indirect ELISA with a TC-F9 cell extract, as previously reported by McLaughlin and Johnson (1984), we found that some antibodies of the panel bind to the alkaline phosphatase present in this extract (unpublished results).

TABLE I

Clasification according to the localization of the antigen recognized by monoclonal antibodies in TC-F9 cells and mouse embryos

| Group | Number of hybridomas | TC-F9 | Oocyte | Implanted Blastocyst | |
|---|---|---|---|---|---|
| | | | | Cell Mass | Trophoblast |
| I | 7 | + | + | + | + |
| II | 0 | + | + | + | – |
| III | 4 | + | + | – | + |
| IV | 4 | + | + | – | – |
| V | 1 | + | – | + | + |
| VI | 7 | + | – | + | – |
| VII | 0 | + | – | – | + |
| VIII | 10 | + | – | – | – |
| Total | 33 | | | | |

*Description of antigen categories*

Group I is represented by 7 monoclonal antibodies that recognize antigens expressed on the oocyte and that during implantation are localized both in the cell mass and the trophoblast. In general, the antigens recognized by these antibodies are present on the surface of the blastomeres throughout the preimplantation and implantation periods (Table II and Figure 3 a, b, e, f and j). None of the antibodies in this group was positive with spermatozoa or with lymphocytes, however, two of them (Mor-F246 and Mor H6B2) were intensely positive with blood red cells. These cell types were selected to determine the localization and specificity of the antigens recognized by these antibodies. We used spermatozoa, because teratocarcinoma cells originate from malignant male germ cells (Martin, 1980); peripheral mouse lymphocytes, since they express histocompatibility antigens on their surface; and erythrocytes, since they express abundant surface oligosaccharide chains, which could be the target for these monoclonals, as has

TABLE II

Localization of antigens with monoclonal antibodies using indirect immunofluorescence

| Group | Hybridoma | TC-F9 | Oocyte | Segmentation | | Implanted Blastocyst | | Other cells | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 2-Cells | 8-Cells | Cell Mass | Trophoblast | Sperm | Erytrocyte | Follicular | Lymphocyte |
| I | Mor-F2A6 | + | + | + | + | + | + | – | + | – | – |
| | Mor-H2A6 | + | + | + | + | + | + | – | + | – | – |
| | Mor-G6D2 | + | + | + | + | + | + | – | – | – | – |
| | TC-3B4 | + | + | +/– | ND | + | + | – | ND | ND | – |
| | TC-3G6 | + | + | +/– | – | + | + | – | – | ND | – |
| | TC-4B10 | + | + | ND | + | + | + | ND | ND | ND | – |
| | TC-5B7 | + | + | + | +/– | + | +/– | – | – | – | – |
| III | TC-2D10 | + | + | + | + | – | + | – | – | – | – |
| | TC-3F4 | + | + | + | + | – | + | – | – | – | – |
| | TC-4E5 | + | + | – | +/– | – | + | – | – | – | – |
| | TC-4F5 | + | +/– | + | +/– | – | +/– | – | – | – | – |
| IV | TC-1C4 | + | + | + | + | – | – | – | – | – | – |
| | TC-1G4 | + | + | ND | – | – | – | ND | ND | ND | – |
| | TC-2H12 | + | +/– | ND | – | – | – | – | – | – | – |
| | TC-4A11 | + | +/– | – | – | – | – | – | – | – | – |
| V | TC-5C7 | + | – | + | + | + | + | – | – | – | – |
| VI | TC-3H5 | + | – | – | – | +/– | – | – | – | – | – |
| | TC-3D4 | + | – | – | + | + | – | – | – | – | – |
| | TC-4B7 | + | – | – | + | + | – | – | – | – | – |
| | TC-5H10 | + | – | – | +/– | + | – | – | – | – | – |
| | TC-6F4 | + | – | – | +/– | + | – | – | – | – | – |
| | TC-8B4 | + | – | – | – | + | – | – | – | – | – |
| Controls | | | | | | | | | | | |
| NSO/2 Supenatant | | – | – | – | – | – | – | – | – | – | – |
| Only 2nd antibody | | – | – | – | – | – | – | – | – | – | – |
| Rat serum against TC-F9 cells | | + | + | + | + | + | + | + | + | +/– | +/– |

ND: not determined; (+) intense fluorescence; (+/–) weak fluorescence; (–) negative fluorescence.

been already reported (Draver *et al*, 1985). Hamasima *et al*, (1985) described an antigen with the characteristics of Group I, although they did not state whether the F2 monoclonal antibody reacted with oocytes.

The molecules recognized by monoclonal antibodies of Group I are not the best to define a stage-specific antigen or cell lineage markers. However, antigens of these groups would participate in the immunologic recognition of the mother by the fetus, as suggested by Wiley and Calarco (1975). Indeed, using indirect immunofluorescence on ovary and oviduct sections, we found an intense reactivity on the oocytes and in some regions of the oviductal epithelium (Figure 3 a, b, c and d).

In our classification, Group II should include the antigens expressed on the oocyte that are later recognized exclusively in the cell mass, however, we did not obtain antibodies with these characteristics. Other authors have described such antigens using rabbit serum against mouse teratocarcinoma cells (Edidin and Gooding, 1975) and blastocyst (Moskalewski and Koprowsky, 1972). Shevinsky *et al* (1982), immunizing with 4- to 6-cell mouse embryos, obtained a monoclonal which recognizes an antigen called SSEA-3 (stage specific embryonic antigen-3) which is present on the oocytes and has been widely used to study the endodermal lineage of the mouse embryo. However, it would not define properly a

stage-specific antigen since it reacts with other tissues, both embryonic and adult, localized in cells of the mouse renal tubules (Fox *et al*, 1984) and in erythrocytes (Damjanov *et al*, 1982).

Group III consists of 4 monoclonal antibodies which bind to antigens expressed in the oocyte and later in the implanted blastocyst, which are localized only in the trophoblast (Table II and Fig 3k). Randle (1982) obtained a monoclonal antibody against teratocarcinomas which is first recognized in the cytoplasm of the unfertilized egg and later expressed on the cell surface of the first segmentation stages as well as in the oviduct and uterus epithelium. When dissociating blastomeres of the advanced morula, the antigen is expressed in the larger cells whereas its expression is reduced or absent in the smaller cells. The same author, in blastomere reaggregation studies, has demonstrated that the smaller blastomeres contribute significantly to the formation of the cell mass while the larger ones are only found in the trophoblast. The results suggest that the antigen described by Randle (1982) and the antigens recognized by our monoclonals – especially TC-2D10 and TC-3F4 for intensity reactivity– are valuable tools as cell lineage markers for the study of the trophoblast cells of the early mammalian embryo. It must be kept in mind that morphological and biochemical studies disclose remarkable differences between the cell mass and the trophoblast, but no fundamental differences have been described between presumptive cell mass and presumptive trophoblast.

On the other hand, antigens recognized by Group III antibodies could participate in the mechanisms of materno-fetal interaction during implantation. Since these mechanisms are not yet known, it is crucial to identify the molecules involved, particularly in trophoblastic tissue, that undergo changes in several cell surface parameters –such as cell surface charge, lectin binding properties and glycoprotein expression– at late pre-implantation and implantation stages (Denker, 1990). Loke *et al* (1990) pointed out that cell surface antigens expressed by the trophoblast –such as MHC-related antigens and blood group related antigens– may influence the interactions of these cells with maternal uterine tissues. Recently, Weitlauf and Knisley (1992) using anti-LAMP-1 and LAMP-2 monoclonal antibodies against lysosome-associated membrane glycoproteins, demonstrated that both monoclonals react with preimplantation mouse embryos from the 1-cell stage onwards and thereafter in the trophoblast; however, the reactivity of LAMP-2 disappears when implantation is delayed, suggesting a functional role for the antigen recognized by LAMP-2.

Group IV consists by 4 monoclonal antibodies, which react to antigens expressed in the oocytes but that are not recognized in the cell mass or in the trophoblast of the implanted blastocyst (Table II) suggesting that they could be antigens remaining from oogenesis which would disappear thereafter.

Group V consists of 1 monoclonal antibody, termed TC-5C7, which recognizes one antigen expressed during segmentation. They are neither expressed in the oocyte nor in the implanted blastocyst and they are present both in the cell mass and in the trophoblast (Table II). A number of antigens that are localized in the same manner as those in Group V have been described, using rabbit serum against blastocysts (Wiley and Calarco, 1975; Johnson and Calarco, 1980a) and teratocarcinoma cells (Artzt *et al*, 1973; Ducibella, 1980) or by means of monoclonal antibodies from rat (Kemler *et al*, 1977) or mouse (Fenderson *et al*, 1983) against teratocarcinoma cells. ECMA 2 and ECMA 3 monoclonals have allowed to define a stage-specific antigen which would be expressed exclusively at the mouse early developmental stage. The antigenic determinants recognized by these monoclonals are oligosaccharides present in embryoglycan (Muramatsu *et al*, 1979; 1983). On the other hand, monoclonals C5 y C6 from Fenderson *et al*, (1983), react with preimplantation mouse embryo antigen, pre-treated with neuraminidase; however such treatment is not required to reveal the presence of these determinants in post-implantation embryos. Several additional monoclonal antibodies that detect embryonic cell surface antigens also seem to recognize carbohydrate specificities. This situation

Biol Res 26: 441-452 (1993)

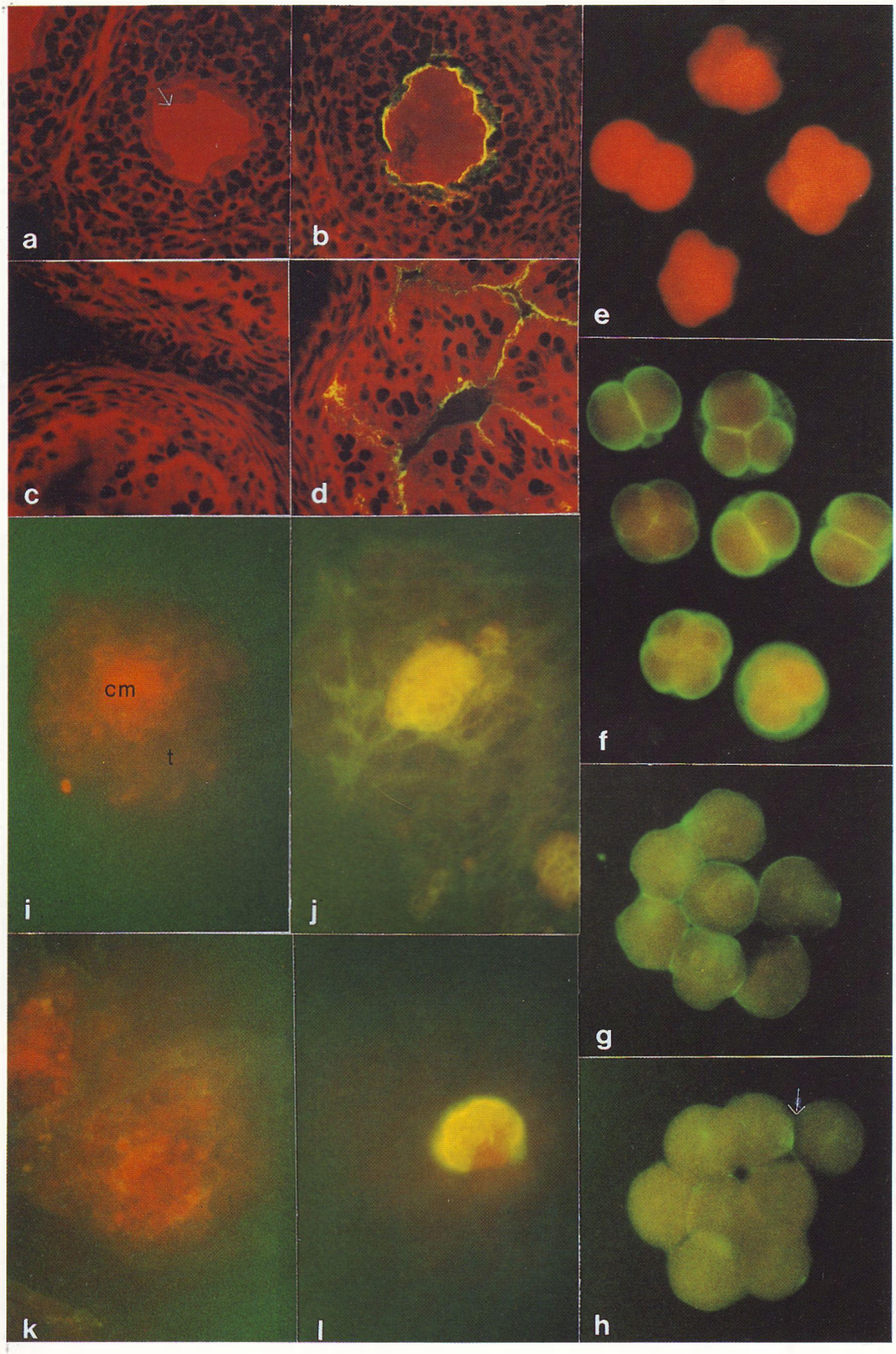

Fig 3: Localization of antigens recognized by antibodies from different Groups using indirect immunofluorescence. **a**, Control. Ovary section incubated only with the conjugate; **b**, Ovary section, incubated with Mor-G6D antibody (Group I). Note the intense fluorescence on the whole oocyte surface and the lack of reaction in follicular cells, x 200; **c**, Control. Oviduct section incubated only with the conjugate; **d**, Oviduct section incubated with Mor-F2A6 antibody (Group I). Note the intense fluorescence on the surface of the lumen epithelium, x 200; **e**. Control, 2-4 cell embryos incubated only with the conjugate; **f**, 2-4 cell embryos incubated with the Mor-G6D2 antibody (Group I). Intense fluorescence is observed on the whole surface of the blastomeres, x 200; **g**, Non compacted morula incubated with TC-4B7 antibody (Group VI). Slight fluorescence is observed on the whole surface of the blastomeres; **h**. Compacted morula incubated with the same antibody as in g. Reactivity is restricted to some contact zones between blastomeres (arrow). For a better observation, morulae in g and h were pressed between slides, x 450; **i**, Control. *In vitro* implanted blastocyst, incubated only with the conjugate. Cell mass (cm) and the trophoblast (t) are clearly seen; **j**. Embryo in a similar stage as in i, incubated with TC-4B10 antibody (Group I). Intense fluorescence on the surface of the cell mass and on the trophoblast; **k**, *In vitro* implanted blastocyst incubated with TC-2D10 antibody (Group III). No fluorescence is observed on the cell mass, however, slight fluorescence is seen on the whole contour of the giant trophoblastic cells; **l**, *In vitro* implanted blastocyst incubated with TC-5H10 (Group VI), intense fluorescence is observed on the cell mass and lack of reactivity in the trophoblast, x 250.

might suggest that either glycosylation enzymes or their appropriate acceptor molecules are stage-specific gene products in early embryogenesis. In this context, it is pertinent to note that antigens associated with the T/t-complex have been demonstrated to involve carbohydrate determinants (Webb, 1983; Silver, 1986).

Group VI is formed by 7 monoclonals against antigens that appear during cleavage and that, during implantation, are exclusively expressed in the cell mass (Table II, Figure 3 g, h and l). It is interesting to note that only Group VI antigens appear at the 8-cell stage, when compaction occurs. In some cases, as with monoclonals TC-3D4, TC-4B7 and TC-5H10, immunofluorescence is observed on the contour of blastomeres of precompacted 8-cell morulae, however, as compaction progresses, immunofluorescence is recognized only in the contact zones and internal blastomeres (Figure 3 g and h). These results suggest a predetermined cleavage pattern in the embryo with the outcome of the localization of these antigens only in the cell mass. Gaunt (1985) found cell surface antigens with such distribution, using rat monoclonals against PC13 teratocarcinoma cells. In a similar fashion to Group III antigens, Group VI antigens are valuable tools as cell lineage markers of the inner cell mass of early embryos. It is not possible to predict if any of the antigens recognized by Group VI monoclonals are stage-specific. Studies with embryonary and adult tissues are required; however, it is worthwhile to mention that none of the monoclonal of the panel affects compaction or blastocyst formation when added to the tissue culture medium. Until now, only monoclonals that react with cell adhesion molecules (CAM) affected the pre-implantation development. It is the case of monoclonals against uvomorulin, present in fetal and adult epithelial tissues (Yoshida-Noro *et al*, 1984; Kemler and Ozawa, 1989) hence, they do not define a stage-specific antigen.

Other authors, using mouse monoclonals against teratocarcinoma cells or mouse spleen cells, found antigens recognized in mouse preimplantation development that are also found in differentiated tissues. The Forssman antigen, a glycolipid, is found in brain and lymphoid tissue (Stern *et al*, 1978: Willison and Stern, 1978) and the SSEA-1 antigen (Solter and Knowles, 1978) is found in the epithelium of oviduct, uterus and epididymis, brain and renal tubules (Fox *et al*, 1981) as well as in cell substratum adhesion (Ozawa *et al*, 1985).

Group VII antigens include antibodies that recognize antigens whose expression is initiated during segmentation and that, during implantation, are only expressed in the trophoblast (Table I). We did not find antigen with such characteristics. Their absence could not be haphazard and suggests that the determination of the trophoblast –the first differentiated embryonic tissue– occurs during the maturation of the oocyte before segmentation. This hypothesis is supported by the results of our work and of others who have described antigens that are localized in the oocyte and that subsequently, during implantation, are recognized only in the

trophoblast (Group III). If this hypothesis is correct, molecules recognized by the antibodies would be localized in the peripheral cells of the morula, generating the regional specification involved in trophoblast differentiation. Hence, antigens recognized by Group VI monoclonals would segregate towards the inner cells of the morula. A model of this cell surface antigen segregation is presented in Figure 4. The results do not permit us to conclude *a priori* whether Group III antigens are actively synthesized by the embryo or whether the antigens are synthesized only during oogenesis and are carried through the blastomeres plasma membrane until they are diluted out or replaced at the blastocyst stage.

*Conclusions*

The existence of trophoblastic antigens regionally specified since oogenesis, does not contradict the models proposed to explain early mammalian development (Tarkowski and Wrobleswska, 1967; Izquierdo, 1977; Izquierdo and Becker, 1982; Johnson *et al*, 1986) and could provide useful tools to study the expression and regulation of the spatial information of the egg during segmentation. One of the drawbacks of the model outside-inside proposed by Tarkowski and Wrobleswska (1967), is that it fails to explain how blastomeres «know» about their position within the morulae. Our results suggest that part of this information would be encoded in the oocyte plasma membrane. The mechanisms involved in the translation of such information could be a predetermined cleavage pattern, as proposed by Gueth-Hallonet and Maro (1992) or, on the other hand, the regionalization of the same components in the plasma membrane as proposed by Izquierdo (1977, 1986, 1991). This phenomenon has been widely demonstrated in mouse, rat and hamster preimplantation embryos, developed either *in vivo* or *in vitro* through cytochemical techniques. The regionalized distribution of some extrinsic proteins of the plasma membrane such as alkaline phosphatase (Izquierdo *et al*, 1980; Lois and Izquierdo, 1984) and 5'-nucleotidase (Izquierdo and Ebensperguer, 1982) has been reported. This regionalization can also be observed using lectins and lipid analogous (Handyside *et al*, 1987).

The spatial segregation of cell surface antigens shows that during mammalian early development (although no differences are recognized among cells, since they are morphogenetically equivalent) there exists a spatial order, recognized by differences between cell parts, which would allow to

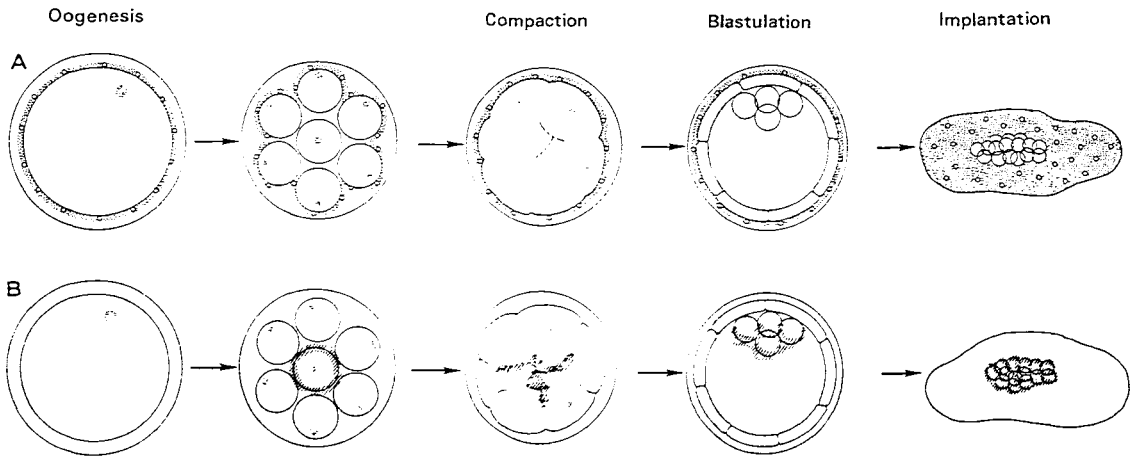


**Fig 4**: Model of segregation of cell surface antigens during segmentation of the mouse preimplantation embryo. A. Molecules recognized by Group III antibodies (white and black dots). Cell surface antigens are already expressed at oogenesis; after fertilization, during cleavage, they localize exclusively on the morula peripheral cells, thus generating the positional information for trophoblastic cell differentiation. B. Molecules recognized by Group VI antibodies (bars). Cell surface antigens whose expression starts during segmentation; during cleavage, they are sequestered in the inner morula cells, generating the positional information for inner cell mass differentiation.

change from a homogeneous spatial distribution to a heterogeneous one, the requisite for explaining the cell differentiation observed in the blastocyst. Finally, it must be stressed that the segregation of mammalian egg surface antigens as described here, could be homologous to the distribution of cytoplasmic localizations observed in some invertebrate eggs (Davidson, 1976).

ACKNOWLEDGMENTS

This article is dedicated to the memory of Prof Luis Izquierdo. We are most grateful to Ms Mónica Imarai, Viviana Cachicas and Ximena Soza for their valuable help, to Mr Juan Aguayo for editing the manuscript, to Mr Ariel Ahumada, Dr Roxana Pey and Dr Arturo Yudelevich for helpful review of the manuscript, to Mr Pablo De Ioannes for computer assistance, to Mr Adolfo Jamett for expert technical assistance, to Ms Drina Acuña, for secretarial assistance, and Ms Ignacia Aguirre for the preparation of the manuscript.

This research was partially supported by FONDECYT grants 0838/91 and 577-89, and Grant 3-P-86-1018-02 from International Development Research Center (IDRC).

REFERENCES

ANDERSON DJ, JOHNSON PM, ALEXANDER NJ, JONES WR, GRIFFIN PD (1987) Monoclonal antibodies to human trophoblast and sperm antigens: Report of two WHO-sponsored workshops. J Reprod Immunol 10: 231-257

ARTZT K, DUBOIS P, BENNETT D, CONDAMINE H, BABINET C, JACOB F (1973) Surface antigens common to mouse cleavage embryos and primitive teratocarcinoma cells in culture. Proc Natl Acad Sci USA 70: 2988-2992

BABINET C, CONDAMINE H (1980) Antibodies as tools to interfere with developmental processes. In: JOHNSON MH (ed) Development in Mammals. Amsterdam: Elsevier. pp 267-305

BENNETT D (1975) The T-locus of the mouse. Cell 6: 441-454

BEUG H, GERISCH G, KEMPFF S, RIEDEL V, CREEMER G (1970) Specific inhibition of cell-contact formation in *Dictyostelium* by univalent antibodies. Exp Cell Res 63: 147-158

BEUG H, KATZ FE, STEIN A, GERISCH G (1973) Quantitation of antigenic membrane sites relating to cell aggregating *Dictyostelium* cells by use of tritiated univalent antibody. Proc Natl Acad Sci USA 70: 3150-3154

BIGGERS JA, WHITTEN WK, WHITTINGHAM DG (1971) The culture of mouse embryos *in vitro*. In: DANIEL JE, FREEMAN WH (eds) Methods in Mammalian Embryology. pp 86-115

DAMJANOV I, FOX N, KNOWLES BB, SOLTER D, LANGE PJ, FRALEY EE (1982) Immunohistochemical localization of murine stage-specific embryonic antigens in human testicular germ cell tumors. Am J Pathol 108: 225-230

DAVIDSON EH (1976) Gene activity in early development. New York: Academic Press

DENKER HW (1990) Trophoblast-endometrial interactions at embryo implantation: A cell biological paradox. Trophoblast Res 4: 3-29

DRAVER P, POKORNA Z, VOJTISKOVA M (1985) Antibodies against surface antigens common to spermatogenic and F9 teratocarcinoma cells in mice immunized with blood group substances from human saliva. Exp Clin Immunogenet 2: 174-182

DUCIBELLA T (1980) Divalent antibodies to mouse embryonal carcinoma cells inhibit compaction in the mouse embryo. Dev Biol 79: 356-366

EDIDIN M, GOODING LR (1975) Teratoma-defined and transplantation antigens in early mouse embryos. In: SHERMAN MI, SOLTER D (eds) Teratomas and Differentiation. New York: Academic Press. pp 109-120

FEIZI T (1985) Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. Nature 314: 53-57

FENDERSON BA, HAHNEL AC, EDDY EM (1983) Immunohistochemical localization of two monoclonal antibody-defined carbohydrate antigens during early murine embryogenesis. Dev Biol 100: 318-327

FERNANDEZ MS, IZQUIERDO L (1980) Blastocoele formation in half and double embryos. Anat Embryol 160: 77-81

FOX N, DAMJANOV I, MARTINEZ-HERNANDEZ A, KNOWLES BB, SOLTER D (1981) Immunohistochemical localization of the early embryonic antigen (SSEA-1) in postimplantation mouse embryos and fetal and adult tissues. Dev Biol 83: 391-398

FOX N, DAMJANOV I, KNOWLES BB, SOLTER D (1984) Stage-specific embryonic antigen SSEA-3 as a marker of visceral extraembryonic endoderm. Dev Biol 103: 263-266.

GAUNT SJ (1985) *In vivo* and *in vitro* cultured mouse preimplantation embryos differ in their display of a teratocarcinoma cell surface antigen: Possible binding of an oviduct factor. J Embryol Exp Morphol 88: 55-69

GIUDICE G (1962) Restitution of whole larvae from disaggregated cells of sea urchin embryos. Dev Biol 12: 233-247

GOLDSCHNEIDER I, MOSCONA AA (1972) Tissue-specific cell surface antigens in embryonic cells. J Cell Biol 53: 435-449

GRAHAM CF (1977) Teratocarcinoma cells and normal embryogenesis. In: SHERMAN M (ed) Concepts in Mammalian Embryogenesis. England: MIT Press. pp 315-394

GUETH-HALLONET C, MARO B (1992) Cell polarity and cell diversification during early mouse embryogenesis. Trends Genet 8: 274-279

HAMASIMA N, SETO M, MOMOI T, TAKAHASHI T (1985) Serological analysis of early mouse embryo with rat monoclonal antibodies produced against mouse teratocarcinoma cells. Differentiation 28: 260-267

HANDYSIDE AH, EDIDIN M, WOLF D (1987) Polarized distribution of membrane components on two-cell mouse embryos. Roux's Arch Dev Biol 196: 273-278

HARLOW E, LANE D (1989) Monoclonal antibody. A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory. pp 471-510

IZQUIERDO L (1977) Cleavage and differentiation. In: JOHNSON MH (ed) Development in Mammals. Amsterdam: Elsevier. 2: 99-118

IZQUIERDO L, LOPEZ T, MARTICORENA, P (1980) Cell membrane regions in preimplantation mouse embryos. J Embryol Exp Morphol 59: 98-102

IZQUIERDO L, BECKER MI (1982) Effect of $Li^+$ on preimplantation mouse embryos. J Embryol Exp Morphol 67: 51-58

IZQUIERDO L, EBENSPERGUER C (1982) Cell membrane regionalization in early mouse embryos as demonstrated by 5'-nucleotidase activity. J Embryol Exp Morphol 67: 115-126

IZQUIERDO L (1986) Desde la especificación regional a la diferenciación celular en el desarrolllo preimplantacional de mamíferos. Arch Biol Med Exp 19: 279-300

IZQUIERDO L (1991) The beginning of mammalian development. Arch Biol Med Exp 24: 215-228

JOHNSON LV, CALARCO PG (1980a) Immunological characterization of embryonic cell surface antigens recognized by antiblastocyst serum. Dev Biol 79: 208-223

JOHNSON LV, CALARCO PG (1980b) Mammalian preimplantation development: The cell surface. Anat Record 196: 201-219

JOHNSON MH, CHISHLOM JC, FLEMING TP, HOULISTON E (1986) A role for cytoplasmic determinants in the development of the mouse early embryo. J Embryol Exp Morph 97: 97-121.

KEMLER R, OZAWA M (1989) Uvomorulin-catenin complex: cytoplasmic anchorage of a $Ca^{++}$ dependent cell adhesion molecule. BioEssay 11: 88-91

KEMLER R, BABINET C, EISEN H, JACOB F (1977) Surface antigen and early differentiation. Proc Natl Acad Sci USA 74: 4449-4452

KEMLER R, MORELLO D, JACOB F (1979) Properties of some monoclonal antibodies raised against mouse embryonal carcinoma cells. In: DOUARIN LE (ed) Cell lineage, stem cells and cell determination. Amsterdam: Elsevier. pp 101-103

KOHLER G, MILSTEIN C (1975) Continuous cultures of fused secreting antibody of predefined specificity. Nature 256: 495-497

LOIS P, IZQUIERDO L (1984) Cell membrane regionalization and cytoplasm polarization in the rat early embryo. Roux's Arch Dev Biol 193: 205-210

LOKE YW, KING A, GRABOWSKA A (1990) Antigenic expression by migrating trophoblast and its relevance to implantation. A review. Trophoblast Res 4:191-207

MARTIN GR (1980) Teratocarcinomas and mammalian embryogenesis. Science 209: 768-776.

McCLAY DR, CHAMBERS AF (1978) Identification of ectoderm and endoderm from sea urchin *Pluteus larvae* and demonstration of germ layer specific antigens. Dev Biol 71: 289-296

MCLAUGHLIN PJ, JOHNSON PM (1984) A search for human placental type alkaline phosphatases using monoclonal antibodies. In: STIGBRAND T, JOHNSON PM (eds) Human Alkaline Phosphatase. New York: Alan Liss Inc. pp 67-78

MONOD J, JACOB F (1961) Teleonomic mechanism in cellular metabolism, growth and differentiation. Cold Spring Harbor Symp 26: 398-401

MOSCONA AA (1968) Cell aggregation: Properties of specific cell-ligands and their role in the formation of multicellular systems. Dev Biol 18:250-267

MOSKALEWSKI S, KOPROWSKY H (1972) Presence of egg antigen in immature oocytes and preimplantation embryos. Nature 237: 167-168

MURAMATSU H, GACHELIN G, DEMONNEVILLE M, DELARBRE C, JACOB F (1979) Cell surface carbohydrates of embryonal carcinoma cells: polysaccharidic side chains of F9 antigens and of receptors to two lectins, FBP and PNA. Cell 18: 183-191

MURAMATSU H, ISHIHARA H, MIYAUCHI T, GACHELIN G, FUJISAKI T, TEJIMA S, MURAMATSU T (1983) Glycoprotein-bound large carbohydrates of early embryonic cells: Structural characteristics of the glycan isolated from F9 embryonal carcinoma cells. J Biochem 94: 799-810

OZAWA M, MURAMATSU T, SOLTER D (1985) SSEA-1, a stage specific embryonic antigen of the mouse, is carried by the glycoprotein involved in teratocarcinoma cell adhesion to substratum. Exp Cell Res 158: 127-143

RANDLE BJ (1982) Cosegregation of monoclonal antibody reactivity and cell behaviour in the mouse preimplantation embryo. J Embryol Exp Morph 70: 261-278

RUTISHAUSER U, EDELMAN GM (1978) Transmembrane control and cell surface recognition. In: Differentiation and Development. Miami Winter Symposia 15: 211-233

SHEVINSKY LH, KNOWLES BB, DAMJANOV I, SOLTER D (1982) Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. Cell 30: 697-705

SILVER LM (1986) Mouse t haplotypes. Annu Rev Genet 19: 179-208

SOLTER D, KNOWLES B (1978) Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc Natl Acad Sci USA 75: 5565-5569

STERN PL, WILLISON KR, LENNOX E, GALFRE G, MILSTEIN C, SECHER D, ZIEGLER A (1978) Monoclonal antibodies as probes for differentiation and tumor-associated antigens: a Forssman specificity on teratocarcinoma stem cells. Cell 14: 775-783

TARKOWSKI AK, WROBLESWSKA J (1967) Development of blastomeres of mouse eggs isolated at the 4- and 8-cells stage. J Embryol Exp Morph 18: 155-180

THIERY JP, BRACKENBURY R, RUTISHAUSER V, EDELMAN GM (1977) Purification of a cell-adhesion molecule from neural retina. J Biol Chem 252: 6841-6845

WEBB CAG (1983) Glycoproteins on gamete and early embryos. In: JOHNSON MH (ed) Development in Mammals. Amsterdam: Elsevier 5: 155-185

WEITLAUF HM, KNISLEY K (1992) Changes in surface antigens onpreimplantation mouse embryos. Biol Reprod 46: 811-816

WILEY LM, CALARCO PG (1975) The effects of anti-embryo sera and their localization on the cell surface during mouse preimplantation development. Dev Biol 47: 407-418

WILLISON KR, STERN PL (1978) Expression of a Forssman antigenic specificity in the preimplantation mouse embryo. Cell 14: 785-793

YOSHIDA-NORO C, SUZUKI N, TAKEICHI M (1984) Molecular nature of the calcium dependent cell-cell adhesion system in mouse teratocarcinoma and embryonic cells studied with a monoclonal antibody. Dev Biol 101: 19-27