

## ENDO A cyokeratin expression in the inner cell mass of parthenogenetic mouse embryos

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**ABSTRACT** During the preimplantation period of development, the first cellular polarization and diversification of the mouse embryo occurs. This process starts at the eight-cell stage and is directly driven by the cytoskeleton. Cell polarization finally leads to the first embryonic epithelium, the trophoctoderm, characterized by the presence of cytokeratins. It has not been described whether genomic imprinting, an epigenetic modification of certain genes depending on the parent-of-origin, affects preimplantation development. However, implantation is one of the steps in which an exceptionally high mortality rate is observed in mouse parthenogenetic embryos, a phenomenon that may be influenced by a deficiency in trophoctoderm differentiation. To assess this possibility we analyzed the expression of various cytoskeletal proteins in late preimplanted embryos. No differences were observed in the expression of microtubules and microfilaments, but surprisingly, the undifferentiated cells of the parthenogenetic inner cell mass showed distinct cytokeratin staining. This anomalous cytoskeleton expression may be considered as one of the earliest manifestation described to date of the effect of genomic imprinting in development.

**KEY WORDS:** *cytoskeleton, parthenogenetic mouse embryo*

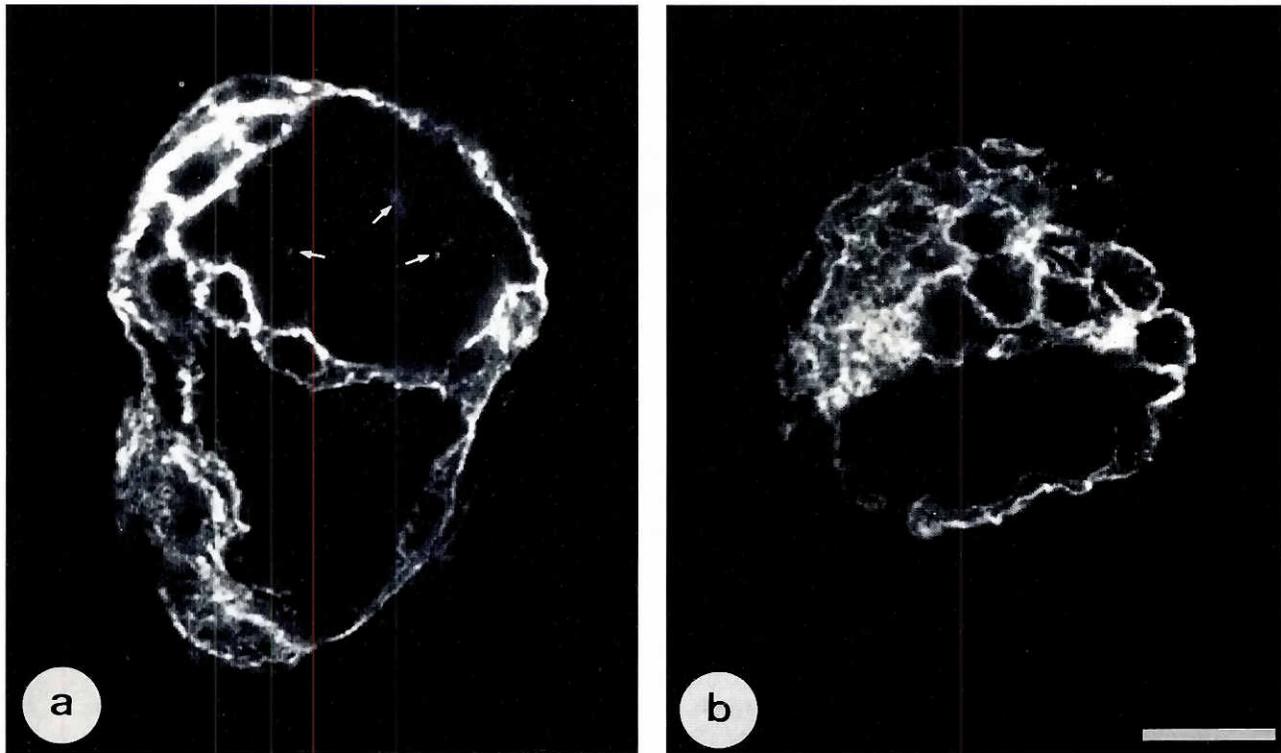
The first mouse embryonic diversification starts during the morula stage when a group of cells differentiates into the trophoctoderm — the first embryonic epithelium — while the other group of cells differentiates into the inner cell mass (ICM). Trophoctoderm generates the blastocoel fluid and simultaneously the ICM differentiates into primitive endoderm and primitive ectoderm (ICM core). Trophoctoderm, endoderm and ICM differ in their properties, developmental potential and fate. The ICM will generate the embryo proper and the trophoctoderm and the primitive endoderm will give rise mainly to extraembryonic membranes. This cellular differentiation process involves changes in gene expression of certain proteins and cellular structures such as cell adhesion molecules, intercellular junctions and cytoskeletal intermediate filaments restricted to the epithelial layers (Fleming and Johnson, 1988). The only intermediate filaments found in this stage are a set of cytokeratins (ENDO A and ENDO B) which are assembled into filaments in some blastomeres of the embryo as early as the eight-cell stage. However, they undergo a progressive increase in presence, density and organization throughout the morula stage until they become restricted to the trophoctodermal/endodermal tissues, and closely associated with desmosomes (Chisholm and Houliston, 1987; Fleming and Johnson, 1988).

Parthenogenetic mouse embryos complete the preimplantation stage and initiate postimplantation development but fail to differentiate normally. Only a few give rise to 25-somite

embryos, of a smaller size than normal and a very poor development of the trophoblast. This is due to the so-called imprinted genes that make complementary, rather than equivalent, maternal and paternal contributions to the embryonic genome (Surani *et al.*, 1990). No phenotypic differences have been described during the preimplantation period comparing normal and parthenogenetic blastocysts. However, in the preimplantation period the parthenogenetic mortality rate is higher (Varnuza *et al.*, 1993). We compared the expression and distribution of microtubules, microfilaments and cytokeratin filaments in control and parthenogenetic whole mouse embryos and isolated ICM to study whether an abnormal expression or distribution of these proteins may be associated with the high mortality rate.

Our results of whole-mount embryos stained with antibodies against microtubules and microfilaments and observed under confocal microscopy reveal no differences in the expression pattern of these proteins in controls and parthenogenetic embryos (not shown). But surprisingly, parthenogenetic and control embryos do show a different cytokeratin filament distribution: in all parthenogenetic embryos observed, cytokeratin filaments are adjacent to the cell-contact areas both in the trophoctoderm/endoderm epithelia and in the ICM cells (Fig. 1a). The cytokeratin expression in control embryos agrees with previous observations (Chisholm and Houliston, 1987; Lehtonen, 1987; Emerson, 1988) in that cytokeratin filaments are restricted to the

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**Fig. 1. Confocal optical section of a control (a) and parthenogenetic (b) blastocyst. (a)** Note that the ENDO A cytokeratin expression is restricted to the trophoblast and the primitive endoderm. Some positive dots also appear in the ICM (arrows). **(b)** ENDO A cytokeratin expression is shown in all the parthenogenetic cells including ICM and trophoblast. Bar, 25  $\mu$ m.

trophoblast and primitive endoderm with no filaments in the ICM cells (Fig. 1b).

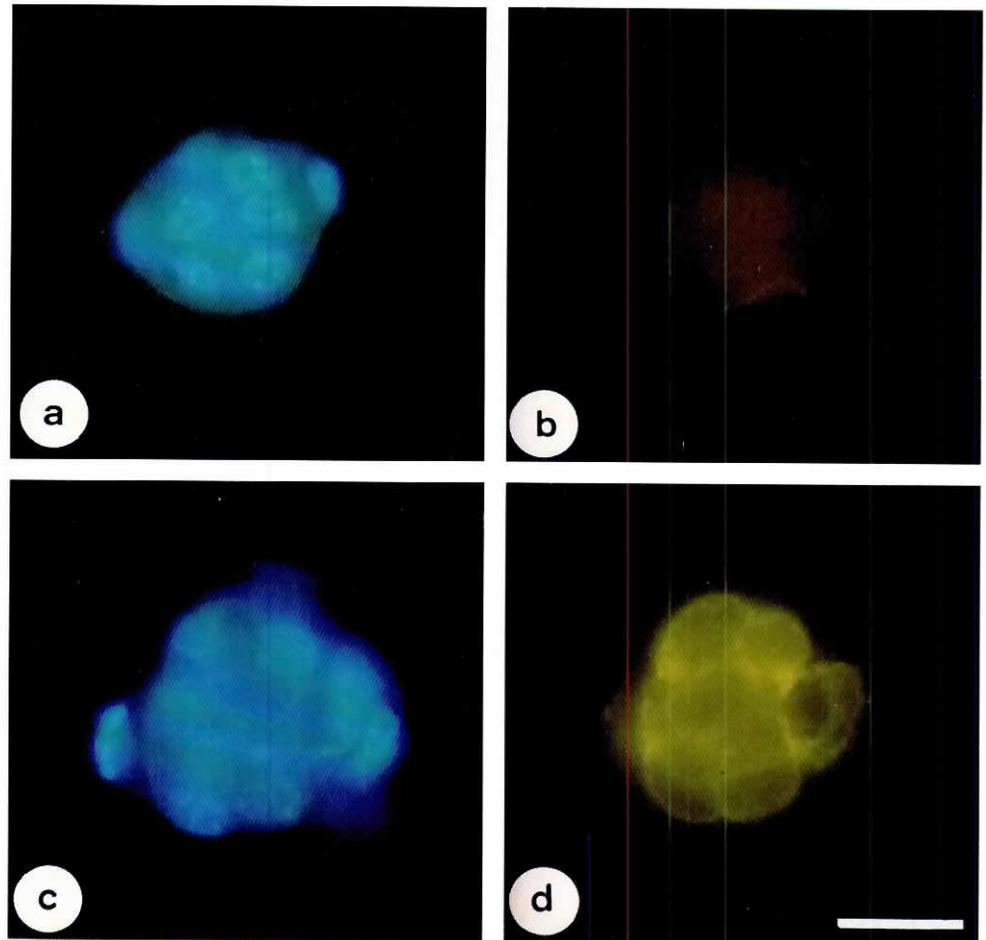
These observations have been confirmed using ICMs isolated by immunosurgery. Parthenogenetic ICMs show cytokeratin positivity underlying the cellular membrane (Fig. 2c) whereas controls are negative, although some slight positivity can be seen in some peripheral cells (Fig. 2a). Although several reports exist describing the destruction of all the trophoblast cells by this technique, exposure time to the antibody is variable, meaning that this residual positivity may be due to cellular debris or partially-lysed cells that have escaped complete disaggregation and still contain some antigen recognized by the TROMA-1 antibody. Chisholm and Houlston (1987) explained the presence of cytokeratin material in some ICM cells as the result of a residual retention in cells that are descendants of the outer cells of the 16-cell morula. However, other authors (Emerson, 1988) have proposed that this material could be an indicator of the initial steps of primitive endoderm formation. A possible explanation for our observations could be the absence of some kind of specific paternally-expressed factor involved in the repression of cytokeratin filament assembly in the ICM, but not in the more differentiated trophoblast. A similar process has been reported by Kay *et al.* (1994), who describe the imprinting and inactivation of the X chromosome as a consequence of the differentiation process. Additionally, it could also be considered that parthenogenetic ICM fail to maintain undifferentiated stem cells. On this point it is interesting to note that Newman-Smith and Werb

(1995) have recently observed that parthenogenote ICM outgrowths differentiate into parietal endoderm probably because they lack a proliferative signal. This epithelial differentiation of parthenogenetic ICM could be responsible for the cytokeratin expression we have observed.

Finally, it also seems that this anomalous cytokeratin expression does not affect the development of the blastocyst. Emerson (1988) disrupted keratin assembly by injecting TROMA-1 antibody and found no obvious effects on the morphological development of the embryos into blastocyst. This would mean that an extensive filament network is not essential for trophoblast differentiation. Similarly, the alteration in the distribution caused by the absence of paternal chromosomes may not have any deleterious effect on the development of preimplanted parthenogenetic embryos, although it might be important in the differentiation of implanted ones.

### Experimental Procedures

Control embryos were obtained from superovulated outbred OF-1 mice using standard methods. Oocytes from non-mated females were activated 20 h after hCG injection by treatment with 5  $\mu$ M calcium ionophore A23187 and  $10^{-4}$  M 1-oleyl-2-acetyl-glycerol (OAG) phorbol ester for 15 min at 37°C in T6 (Uranga *et al.*, 1992). Cytochalasin D (1  $\mu$ g/ml, 5 h) was used to avoid second polar body emission. Embryos were cultured in T6+EDTA (0.04 mM) medium until the blastocyst stage. ICMs from zona-free controls and parthenogenetic embryos were isolated from early blastocysts according to Solter and Knowles (1975). They



**Fig. 2.** ICMs isolated by immunosurgery from fertilized (a-b) and parthenogenetic embryos (c-d). (b) Negative TROMA-1 antibody staining. Note a slight positivity in some peripheral cells; (d) positive TROMA-1 antibody staining in parthenogenetic MCI; (a-c) nuclei stained with DAPI. Also note that parthenogenetic ICM cells are usually bigger than normal ICM control cells. Bar, 20  $\mu$ m.

were cleaned of debris by intense pipetting through a narrow flame-polished pipette. For tubulin staining zona-free full-size blastocysts and ICMs were placed in stainless steel chambers coated with Concanavalin A and extracted with 0.25% Triton X100 in PHEM-taxol buffer, fixed for 30 min in 4% formaldehyde in the same buffer and washed in 50 mM  $\text{NH}_4\text{Cl}$  (Houliston *et al.*, 1987). The same method was used for cytokeratins although fixation was done prior to extraction (Chisholm and Houliston, 1987). In order to label actin filaments, cells were extracted and fixed with 1.8% formol and Triton X100 for 20 min at 4°C in PBS. Immunocytological staining was performed using YL1/2 anti-tubulin antibody (Houliston and Maro, 1989), TROMA-1 monoclonal antibody directed against cytokeratin ENDO A (Kemler *et al.*, 1981) and TRITC-labeled Phalloidin against actin (Sigma). Nuclei and chromosomes were stained with DAPI. Regular fluorescence microscopy and interferential contrast images were obtained with a Leitz DMR microscope. Whole embryos were observed with Sarastro Phoibos 1000 and BioRad MRC 600 confocal scanning laser microscopes at a 488 nm wavelength for fluoresceine and 543 nm for rhodamine.

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