On the control of the trophoblastic giant-cell transformation in the mouse: homotypic cellular interactions and polyploidy

By E. B. ILGREN[1]

From the Departments of Botany and Zoology,
University of Oxford

SUMMARY

Trophoblastic tissues grown under different conditions *in vitro* display distinct patterns of cellular growth. Thus, trophoblast cultured on 'bacteriological grade' plastic surfaces remained in suspension culture as rounded tissue fragments. Such tissues maintained numerous cell contacts and remained, in turn, largely diploid. Trophoblast explanted on a 'tissue-culture grade' substrate formed monolayers. These contained fewer cell contacts and had more giant nuclei than the rounded tissues. Finally, if trophoblast was dissociated and grown as attached single cells, so that cell contact was minimal or absent, the single-cell preparations contained more giant nuclei than tissues grown either as monolayers or in suspension. These results suggest that changes in tissue shape and the number of cell contacts can modify the growth of mouse trophoblast and alter its ability to become giant.

INTRODUCTION

Trophectodermal proliferation is dependent on contact with the inner cell mass (ICM) (Gardner, 1972). Loss of such contact either during normal pre-implantation development (Copp, 1978) or following removal of the ICM (Gardner, 1972) leads to cessation of trophectodermal cell division. Trophecto-dermal cells which remain adjacent to (Copp, 1978) or are secondarily brought into contact with (Gardner, Papaioannou & Barton, 1973) the ICM continue to divide and form the ectoplacental cone and extraembryonic ectoderm follow-ing implantation (Gardner & Papaioannou, 1975). When these postimplantation tissues are isolated microsurgically from the embryo and subsequently grown as cellular monolayers *in vitro* or ectopic grafts, division ceases and the cells become giant (Rossant & Offer, 1977). In contrast, isolated embryonic ecto-derm remains diploid and mitotically active under identical experimental condi-tions (Rossant & Offer, 1977). Such findings suggest that continued contact with ICM derivatives, e.g. extraembryonic endoderm and/or mesoderm, is needed to promote postimplantation trophoblastic proliferation *in vivo*. However, it is

---

[1] *Author's address:* Department of Botany, South Parks Road, Oxford, U.K.

not clear from these studies whether trophoblastic cell division is sustained because of a specific inductive effect or because ICM derivatives maintain these trophectoderm-derived tissues in an appropriate configuration (Rossant & Offer, 1977). Changes in tissue geometry are able to alter DNA synthesis and cell division in various experimental situations (Folkman & Moscona, 1978). Therefore, the aim of the present study was to examine the effect of varying the extent of contact between initially diploid trophoblast cells on their growth *in vitro*. Three experimental conditions were used to influence the degree of intercellular contact between trophectoderm-derived cells. First, trophoblast was grown as intact, rounded tissues in suspension culture in which case the degree of contact between cells was extensive. Secondly, these tissues were explanted on a 'tissue-culture grade' plastic substrate and grown as coherent monolayers wherein intercellular contact would be lessened. Finally, trophoblast was dissociated and cultured as attached single cells whereby contact was either minimal or altogether absent. As controls, embryonic ectodermal tissues which do not form giant nuclei (Rossant & Offer, 1977), were also grown in a manner identical to trophoblast.

MATERIALS AND METHODS

Immediately upon dissection from the embryo some representative samples of embryonic ectodermal, extraembryonic ectodermal, and ectoplacental cone core tissues were examined histologically to check for contaminating endoderm, mesoderm, or giant cells (those with more than four times the haploid ($\bar{c}$) amount of DNA). Other tissue samples were cultured either for 72 or 144 h *in vitro*, subsequently recovered, and then examined in the following way. First, they were studied histologically for evidence of necrosis and differentiation, then cytologically for multinucleate cells, polyploid metaphases (those with more than 40 chromosomes) and changes in mitotic index and cell number, cytophotometrically for fluctuations in nuclear DNA content and autoradiographically for [H³]thymidine-labelled nuclei. The viability of trophoblastic fragments grown in suspension was assessed by examining some tissues either histologically or autoradiographically following 72 h in culture or by transferring others, after 72 h *in vitro*, either to tissue-culture dishes ('secondarily attached' tissues) or beneath the testis capsule. Such transferred tissues were then recovered 72 and 144 h later at which time their nuclear DNA contents and mitotic indices were determined.

(1) *Recovery of embryos and the handling of tissues*:

Details of the mating of mice (CFLP, Anglia Lab. Ltd) and estimating the time of ovulation are described by Copp (1978). Embryos at the 7·5-day primitive streak stage (Snell & Stevens, 1966) were recovered from the uteri of mice and subsequently dissected using the methods described by Rossant &

Offer (1977). The cores of ectoplacental cones were obtained by removing the peripheral giant cells with microneedles made as described by Diacumakos (1973), incubating the tissue in a 0·5 % trypsin-2·5 % Pancreatin (made up in $Ca^{2+}-Mg^{2+}$-free Tyrode's solution) solution for 5 min (26 °C; pH 7·5) and then drawing it through a siliconized micropipette drawn to a bore size of one-half the diameter of the enzyme-treated fragment.

(2) *Tissue culture*

All tissues were cultured in the alpha-modification of Eagle's medium (Flow) with 10 % foetal calf serum (Flow), nucleosides (3×10^{-5} M Sigma), $NaHCO_3$ (2·2 g/l), penicillin (60 mg/l), and streptomycin (50 mg/l) in 5 % CO_2 and air. Whole tissue fragments were grown individually in either 50 mm bacterio-logical plastic Petri dishes (Sterilin) or 50 mm tissue-culture dishes (Sterilin) containing 5·0 ml of medium. Single-cell cultures of approximately equivalent cell density (starting cell number given beneath Table 4) were established from embryonic ectodermal (fragments pooled from 15 embryos), extraembryonic ectodermal (from 28 embryos), or ectoplacental cone core (from 10 embryos) tissues that were initially incubated in 2·5 ml of TVP (Bernstein, Hooper, Grandechamp & Ephrussi, 1973) solution (26 °C: pH 7·5; 5–15 min) and then dissociated into single cells by gently drawing the fragments through a fine-bore siliconized Pasteur pipette. The resultant cell suspension was then transferred to a 30 mm plastic Petri dish (Sterilin) containing 2·5 ml of medium and a 22 mm^2 glass coverslip (Chance). Cultures were not disturbed during the first 18 h of growth *in vitro*. Except for undissociated tissues secondarily attached to a tissue-culture-grade substrate, the medium was never changed. Such secondarily attached tissues were placed in fresh medium upon transfer.

(3) *Histological analysis*

Immediately after dissection from the embryo, some tissues were fixed in 25 % glutaraldehyde, embedded in plastic, serially sectioned at 2 μm, and stained with toluidine blue. Other samples were fixed in Bouin's solution, embedded in paraffin wax (56 °C), serially sectioned at 5 μm, and stained with haematoxylin and eosin. Tissues were also prepared after 72 and 144 h *in vitro* using similar histological techniques in order to detect necrosis and assess the degree of cytodifferentiation.

(4) *Mitotic indices, nuclear numbers, and multinucleate cell counts*

Modifications of a method developed by Evans, Burtenshaw & Ford (1972) were used to determine mitotic indices (number of mitoses scored per total cells counted $\times$ 100), to calculate nuclear numbers, and to identify multinucleate cells. After 72 and 144 h *in vitro* tissues were cultured in the presence of 0·04 μg/ml of Colcemid (Grand Is. Biol.) for 75 min, mechanically lifted from the petri surface with the edge of a siliconized micropipette, and transferred to hypotonic

solution (1 % Na$^+$ citrate, pH 8·45, 26 °C) for 3 min (extraembryonic ectoderm), 7·5 min (ectoplacental cone core), or 10 min (embryonic ectoderm). Then, fragments were fixed (three parts methanol/one part acetic acid), dissociated in several drops of 60 % acetic acid (26 °C) on acid-cleaned slides, and stained with 1 % toluidine blue. For embryonic ectodermal tissues, it was difficult to score every nucleus. Therefore, counts were made by scoring at least three areas of a preparation the latter being no more than one-quarter of the width of each slide. In the case of trophoblastic tissues, every nucleus was counted. It was necessary, however, to assume that changes in nuclear number would reflect variations in the number of cells present since cell outlines became blurred after tissue dissociation. This does, in turn, assume that the level of multinucleation was always low. Bi- and multinucleate trophoblastic cells were occasionally found but their exact numbers could not be determined because of enzyme-induced cytoplasmic changes. Such multinucleate, trophoblastic cells were discovered in the following way. First, fragments were recovered after 72 h *in vitro*, transferred to hypotonic, fixative, and then softened in 60 % acetic acid (26 °C; 30–60 sec). Afterwards, tissues were gently squashed beneath a 22 mm² coverslip (no. 00; Chance), the edges of the preparation sealed with nail polish and the slide examined in a contrast microscope (× 40).

(5) *Microdensitometry*

The nuclear DNA contents of freshly dissected embryonic ectodermal, extraembryonic ectodermal, and ectoplacental cone core tissues were determined on fragments that had been dissociated in TVP as described under 'Tissue culture'. Following dissociation, the cell suspension was air-dried on glass slides for 10 min and liver imprints placed beneath the experimentals. Single-cell cultures were prepared for microdensitometry as follows. First, the supernatant was discarded. Then, each coverslip was rinsed briefly with Hank's balanced salt solution (HBSS; 37 °C), twice with TVP (26 °C; pH 7·5 sec), again with HBSS, and finally washed in PBS (Dulbecco A). The coverslip was removed from the petri dish to dry (15 min beneath a lamp). Once dry, both glass slides and coverslip cultures were fixed and Feulgen-stained using a standardized technique. Following culture, intact tissue fragments were also dissociated, fixed and Feulgen–stained. Ectopic grafts performed using the method of Kirby (1963) were processed for microdensitometry in the following way. Either 72 or 144 h after transfer, recipients were killed, the testis capsule bisected next to the haemorrhagic graft site and the trophoblastic nodule placed into Ca²⁺ and Mg²⁺-free HBSS on an acid-cleaned slide. At the same time, the contralateral testis was also bisected and, with the liver control, was smeared on to the slide immediately beneath the experimental. Once dry, all slides were then fixed and Feulgen-stained. Nuclei were scanned in two different ways in order to rule out consistent analytical differences potentially attributable to sampling error and, regardless of method, similar results were always obtained. Thus, all of the

nuclei in at least ten fields were measured but, on occasion, every nucleus in a trophoblast fragment was scanned. This was always carried out with a Vicker's M-85 microdensitometer ($\lambda = 585$ nm).

(6) *Autoradiography*

For autoradiography, methyl [H³]thymidine (TRK-120, Amersham, 20 Ci/ ml; 4×10^{-2} m Ci/ml media) was added to the trophoblastic cultures. After exposure to [H³]thymidine, labelled fragments were processed using two different methods. Some were recovered, dissociated in enzyme, Feulgen-stained, and scanned. Following microdensitometry, coverslips were removed in xylene and each slide processed through the alcohols to distilled water. Other labelled fragments were softened in 60% acetic acid, squashed, and then placed onto blocks of solid CO_2 for 10 min. Once frozen, coverslips were removed from the preparations with the edge of a razor blade. Slides were then immediately air-dried and placed into distilled water. From water, both squash and Feulgen-stained preparations were mounted with stripping film (Kodak AR-10) and set aside for 4 weeks. Finally, slides were developed (Kodak D-19; 10 °C, 5 min), stained with haematoxylin and eosin, and scored. A nucleus was considered to be labelled if covered by five more grains than were found in an equal area of background adjacent to that nucleus. Labelling index (% L.I.) was defined as the number of labelled nuclei scored divided by the total number of nuclei $\times 100$.

(7) *Statistical methods*

A two by two χ^2 contingency test was used to compare the results obtained from analyses performed by microdensitometry (Snedecor & Cochran, 1976). The Fischer exact probability test was used to see if metaphases with more than 40 chromosomes ('polyploid' metaphases) were more frequently found in trophoblastic tissues grown in suspension culture than in those cultured as attached fragments (Siegel, 1956). A Student *t*-test was employed to determine the significance of changes in nuclear numbers with time *in vitro* (Bailey, 1973).

RESULTS

(A) *Morphological observations*

As judged either histologically or visually, all tissues could be isolated from the embryo in pure form. When these embryonic and trophoblastic tissues were explanted on tissue-culture substrates they formed coherent, cellular mono-layers. Conversely, when grown on a 'bacteriological grade' plastic surface, they remained in suspension as floating, spherical tissue fragments. The floating embryonic ectodermal tissues frequently formed a proamniotic cavity and showed signs of morphogenesis *in vitro*, e.g. beating-heart formation. However, trophoblastic tissues grown in suspension culture rarely cavitated. Finally, when the trophoblastic and embryonic ectodermal tissues were dis-

Table 1. *Changes in nuclear DNA content*

Time (h) ...	0	24	72			144		
Culture Conditions*	—	AT d	FL w	AT w	AT d	FL w	AT w	2° AT w
Extraembryonic ectoderm								
Nuclei > 4c̄	0%	39·6%	14·7%	42·2%	86·1%	37·5%	74·8%	71·8%
Max. c̄	4c̄	32c̄	16c̄	16c̄	64c̄	32c̄	32c̄	32c̄
No. nuclei	109	279	2827	2746	165	1382	1813	1239
No. embryos	12	50	16	14	56	14	15	15
Ectoplacental cone core								
Nuclei > 4c̄	0%	87·5%	33%	33%	56·5%	42·0%	81·5%	65·5%
Max. c̄	4c̄	32–64c̄	64c̄	128c̄	128c̄	4c̄	64c̄	64–128c̄
No. nuclei	107	247	1534	2736	277	329	1087	2170
No. embryos	12	20	15	17	20	4	14	14
Embryonic ectoderm								
Nuclei > 4c̄	0%	0%	0·1%	0·6%	—	0%	0%	0·1%
Max. c̄	4c̄	4c̄	4–8c̄	8c̄	—	4c̄	4c̄	4–8c̄
No. nuclei	107	159	1083	350	—	827	605	235
No. embryos	12	12	5	6	—	5	12	12

* Cultures of whole (w) and dissociated (d) tissues grown either as floating (FL) or attached (AT) tissue fragments.

sociated and grown in culture as single cells, they showed little tendency to reaggregate *in vitro*.

(B) *Cytological studies*

(1) *Changes in nuclear DNA content and the number of trophoblastic giant* (> $4\bar{c}$ *cells* (see Table 1). Immediately after isolation from the embryo, all tissues were found to be exclusively diploid (2–$4\bar{c}$). However, after growth *in vitro*, the trophoblastic tissues were always found to contain cells with nuclear DNA contents greater than $4\bar{c}$ and the extent to which these became giant was dependent upon the conditions under which they were grown. Thus, trophoblast cultured as isolated, attached single cells always contained more giant nuclei than either intact explants or whole floating fragments (P always < 0·001). In addition, the whole, rounded extraembryonic ectodermal and ectoplacental cone core tissues grown in suspension always had fewer giant nuclei than the trophoblastic tissue fragments that were explanted on a tissue-culture surface and subsequently cultured as coherent, cellular monolayers (P always < 0·001). In contrast to the trophoblastic derivatives, both intact and dissociated embryonic ectodermal tissues remained diploid (2–$4\bar{c}$) whilst in culture.

(2) *Changes in mitotic index (Table 2)*. All tissues were found to be mitotically active immediately after they were isolated from the embryo. However, following culture, the undissociated trophoblastic tissues ceased cell division whilst, the intact embryonic ectodermal tissues continued to divide. In contrast to the whole fragments, the mitotic index of the dissociated tissues always declined.

(3) *Changes in cell number (Table 3)*. The culture of intact trophoblastic tissues did not show an increase in cell number from one time point to the next (P always < 0·05) except for attached extraembryonic ectoderm during the first 72 h *in vitro* (where P > 0·01). Whole embryonic ectodermal tissues, however, continued to increase in cell number throughout the culture period. The dissociated tissues, in contrast to the whole fragments, always displayed a considerable amount of cell loss after culture.

(4) *Changes in ploidy level (n) and the frequency of polyploid metaphases* (Table 4). Immediately after isolation from the embryo, rare polyploid metaphases were found in the trophoblastic tissues whilst none were seen in embryonic ectoderm. Following 72 h in culture, tetraploid metaphases with 80 chromosomes were most frequently found in the ectoplacental cone core fragments (28·3–41·7 %), less commonly seen in the extraembryonic ectoderm (9·3–21·1 %), but only occasionally noted in the embryonic ectoderm (3·4–5·4 %). After 144 h *in vitro*, tetraploid metaphases were only seen in the cultures of extraembryonic ectoderm (20 %). Finally, octaploid metaphases with 160 chromosomes were only found in extraembryonic ectodermal explants and never in embryonic ectoderm (Fig. 1). Also, changing the conditions under which the trophoblastic tissues were grown did not alter the frequency with which polyploid metaphases

Table 2. *Changes in mitotic index*

Time ...	0	24	72			144		
Culture* Conditions*	—	AT d	FL w	AT w	AT d	FL w	AT w	2° AT w
Extraembryonic ectoderm								
Mitotic index	10·6±1%	5·7±3%	0·3%	0·1%	0·4±0·3%	0·01%	0·4%	0%
No. embryos	7	56	5	5	5	5	5	5
Ectoplacental cone core								
Mitotic index	2·8±1%	ND	0·2%	0·06%	0% / 30%	0·01%	0·01%	0%
No. embryos	6		5	5		5	4	4
Embryonic ectoderm								
Mitotic index	7·4±5%	5·1%	2·4±0·4%	2·4±0·4%	—	1·1±1%	1·0±0·1%	1·3±0·4%
No. embryos	5	15	7	6	—	3	3	4

* Cultures of whole (w) and dissociated (d) tissues grown either as floating (FL) or attached (AT) tissue fragments.

Table 3. *Changes in cell numbers*

(Average number of cells in each tissue fragment. No. of embryos
in parentheses)
Non-dissociated tissues grown as attached (AT) explants and floating
(FL) fragments

Time (h) ...	0	72	
Culture Conditions	—	FL —	AT —
Extraembryonic ectoderm	2051 ± 1094 (17)	2223 ± 760 (6)	4568 ± 826 (5)
Ectoplacental cone core	4158 ± 1390 (10)	3888 ± 417 (8)	4053 ± 637 (5)
Embryonic ectoderm	1976 ± 455 (11)	> 6000 (6)	> 6000 (6)

Time (h)		144	
Culture Conditions	FL —	AT —	2° AT —
Extraembryonic ectoderm	2355 ± 1687 (3)	4868 ± 2185 (4)	ND
Ectoplacental cone core	4541 ± 1329 (3)	2070 ± 551 (4)	ND
Embryonic ectoderm	> 8000 (6)	> 8000 (6)	> 8000 (5)

Dissociated tissues grown as attached single-cell cultures

Time (h) ...	0	24	72
Extraembryonic ectoderm	57 400	3961 ± 281 (56)	1722 ± 230 (112)
Ectoplacental cone core	41 500	3951 ± 24 (30)	2578 ± 707 (30)
Embryonic ectoderm	29 625	2133 (15)	0 (60)

were found in either the extraembryonic ectodermal or ectoplacental cone core cultures (P always > 0·01).

(5) *Presence of multinucleate cells and diplochromosomes.* Diplochromosomes (Fig. 2) and multinucleate cells (Fig. 3) were only found within trophoblastic tissues, never within embryonic ectoderm. Moreover, each multinucleate cell had between 5 and 50 interphase nuclei and rare mitotic figures. A considerable number of interphase nuclei within each multinucleate cell was found to be

Table 4. *Changes in ploidy level (n) and the frequency of polyploid metaphases*

(Numbers in parentheses show total number of metaphases scored.
'd' indicates number of diplochromosomes.)

Time (h)	0	72		144		
		FL	AT	FL	AT	2 ° AT
Culture Conditions	—	(w)	(w)	(w)	(w)	(w)
	—					
% of all metaphases with (40 chromosomes						
Extraembryonic ectoderm	99·8% (2532)	86% (43)	78·9% (19)	100% (1)	76·3% (80)	0% (0)
Ectoplacental cone core	99·6% (891)	76·2% (63)	58·3% (12)	100% (1)	100% (2)	0% (0)
Embryonic ectoderm	100% (505)	93·6% (125)	94·6% (112)	100% (100)	98·3% (117)	100% (100)
% of all metaphases with (80) chromosomes						
Extraembryonic ectoderm	0·2% (2532) 0 d	9·3% (43) 1 d	21·1% (19) 4 d	0% (11) 0 d	20·0% (80) 0 d	0% (0) 0 d
Ectoplacental cone core	0·4% (891) 0 d	29·3% (63) 7 d	41·7% (12) 2 d	0% (1) 0 d	0% (2) 0 d	0% (0) 0 d
Embryonic ectoderm	0% (505) 0 d	3·4% (125) 0 d	5·4% (112) 0 d	0% (0) 0 d	1·7% (2) 0 d	0% (0) 0 d
% of all metaphases with (160) chromosomes						
Extra embryonic ectoderm	0% (2526) 0 d	4·7% (43) 2 d	0% (10) 0 d	0% (1) 0 d	3·7% (80) 3 d	0% (0) 0 d

labelled after exposure to thymidine. However, since the cell outlines of the multinucleates became blurred after autoradiography, the exact number of labelled nuclei could not be determined.

(C) *Tests used to determine the viability of trophoblastic tissues grown in suspension culture*

(1) *Tissues 'secondarily attached' (see '2° AT' in Table 1)* in vitro. After 72 h in suspension culture, some trophoblastic tissues were transferred from 'bacteriological grade' to 'tissue-culture grade' dishes. Following an additional 72 h *in vitro*, these 'secondarily attached' tissues were recovered and found to contain more giant nuclei than those which had been grown in suspension culture for the entire 144 h time course. In contrast to trophoblast, the 'secondarily-attached' embryonic ectodermal tissues never contained giant nuclei.

(2) *Tissues ectopically transferred to the testis capsule.* After 72 h in suspension
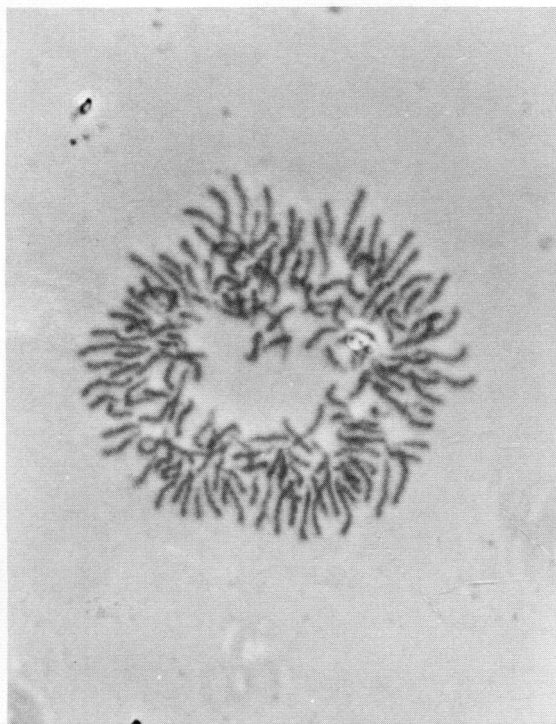
Fig. 1. Octoploid (160 chromosomes) late metaphase/anaphase recovered from an extraembryonic ectodermal tissue fragment after 72 h in suspension culture. Toluidine blue. ($\times$ 2000).

culture some trophoblastic tissues were transferred from 'bacteriological grade' dishes to the testis capsule. The transferred tissues always produced haemorrhagic nodules and each nodule was always found to contain at least one class of giant nuclei larger (e.g. > 64$\bar{c}$) than any found within the 72 h, suspension cultures (Figs. 4 and 5). In addition the contralateral control testes contained only haploid (1–2$\bar{c}$) and diploid (2–4$\bar{c}$) nuclei (Figs. 4 and 5).

(3) *Tissues labelled with tritiated thymidine.* Extraembryonic ectodermal (L.I. = 48·5 %, six embryos) and ectoplacental cone core tissues (L.I. = 43·5 %, six embryos) initially grown in suspension culture for 72 h and then exposed to tritiated thymidine, always contained considerable numbers of labelled nuclei.

(4) *Tissues examined histologically.* Upon histological examination, trophoblastic tissues displayed little evidence of necrosis (Fig. 6). Similarly, histological study of the embryonic ectodermal floating fragments showed occasional dead cells and evidence of cardiomyogenesis and early somite formation.
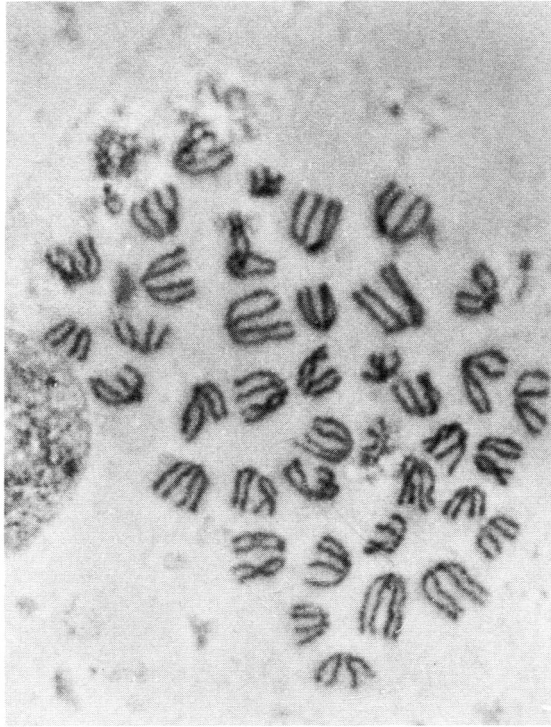
Fig. 2. Diplochromosomes in an endoreduplicated tetraploid (80 chromosomes) metaphase found in an extraembryonic ectodermal tissue fragment after 72 h in suspension culture. Toluidine blue (×2000).

DISCUSSION

Exclusively diploid embryonic ectodermal and trophoblastic tissues have been grown *in vitro* under identical conditions. However, despite their initial similarities, the nuclear DNA contents and mitotic indices, as well as the levels of ploidy and multinucleation of these two types of tissue, consistently differ following culture. Thus, embryonic ectoderm always remains diploid (2–4c̄), continues to divide, and increases in size and cell number after culture. Trophoblast, however, not only fails to divide and increase in cell number but subsequently becomes both polyploid (> 2n) and giant (> 4c̄) following growth *in vitro*. The extent to which trophoblast becomes giant is dependent upon the conditions under which it is grown. Giant nuclei are most frequently found when trophoblastic tissues are cultured as single cells and intercellular contact is either

FIGURE 3

Multinucleate giant cells from (a) extraembryonic ectodermal (×240) and (b) ectoplacental cone core fragments (×400) after 72 h in suspension culture. Mitotic figures in each cell shown in (c) ×720 and (d) ×960 below. Phase contrast. See also Haythorn (1928) for examples of multinucleate giant cells with rare mitoses.
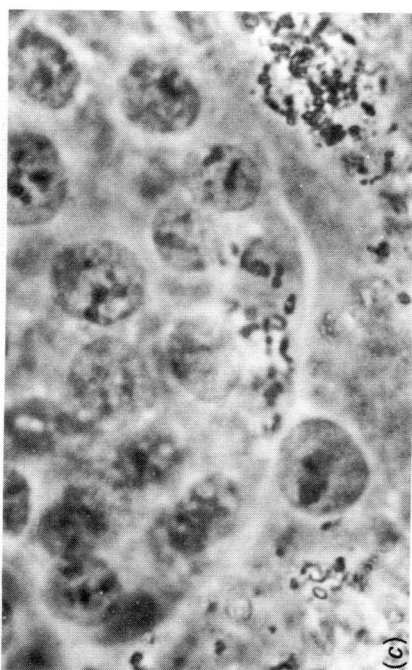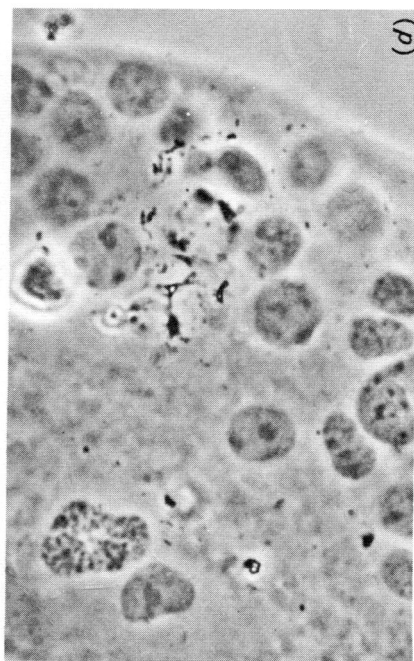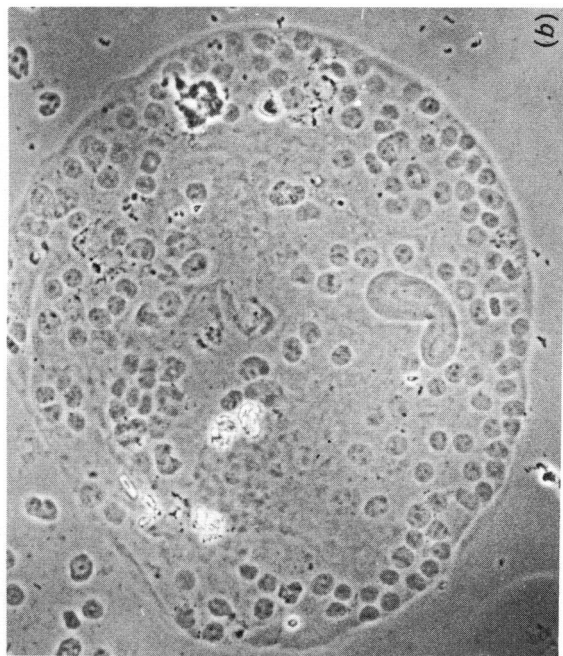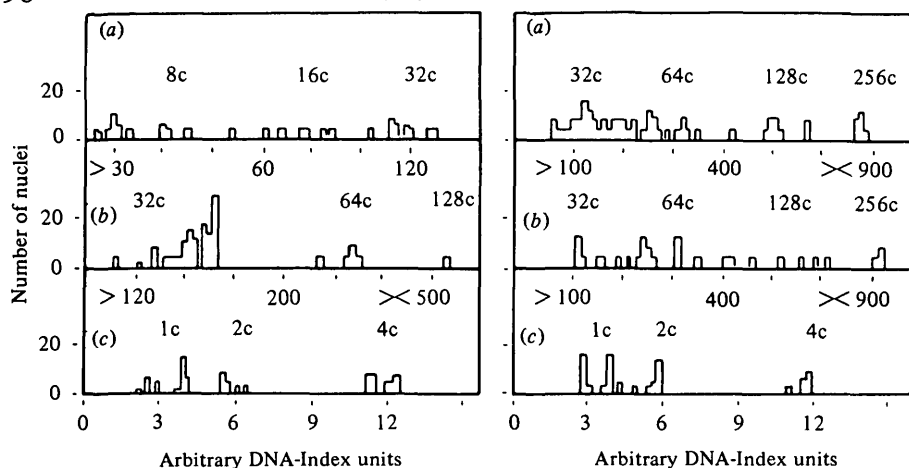
Fig. 4. Nuclear DNA contents of extraembryonic ectodermal cells ectopically transferred to the testis capsule after 72 h in suspension culture and then recovered after another (a) 72 or (b) 144 h beneath the testis capsule, (c) contralateral control testis.

Fig. 5. Nuclear contents of ectoplacental cone core cells ectopically transferred to the testis capsule after 72 h in suspension culture and then recovered after another (a) 72 or (b) 144 h beneath the testis capsule has elapsed. (c) Contralateral control testis.

minimal or absent. Coherent monolayers contain fewer giant nuclei than cultures of dissociated trophoblast. Trophoblastic tissues grown in suspension culture as rounded, cellular fragments maintain numerous cell contacts and remain largely diploid (> 50 % of all nuclei, 2–4c̄). Such rounded trophoblastic tissues are neither growth-arrested nor dying since they contain numerous labelled nuclei, show little histological evidence of necrosis, and continue to endo-reduplicate either in suspension, when returned to a tissue-culture substrate, or upon transfer to the testis capsule. The fact that intact trophoblastic tissues, except in one case, did not change in cell number from one time point to the next and free floating cells were not found in the media in which these undissociated tissues were grown also suggests that cell death and loss were minimal. From these findings it would therefore appear that tissue geometry and the extent of intercellular contact can influence the growth of mouse trophoblast. Nevertheless, even when trophoblastic tissues are grown as three-dimensional, rounded fragments whereby extensive intercellular contact is maintained and most cells remain diploid (2–4c̄), cell division still fails to occur. Therefore, tissue configuration *per se* probably cannot account for the cellular division which takes place within the ectoplacental cone core or the extraembryonic ectoderm *in vivo*. This, in turn, suggests that the presence of ICM derivatives as well as the maintenance of an appropriate tissue configuration may both be required to promote the proliferation of postimplantation mouse trophoblast. However, it is still no known whether these ICM-derived tissues achieve this effect via diffusable signals (Hsu, 1980; Sellens & Sherman, 1980; Patterson,
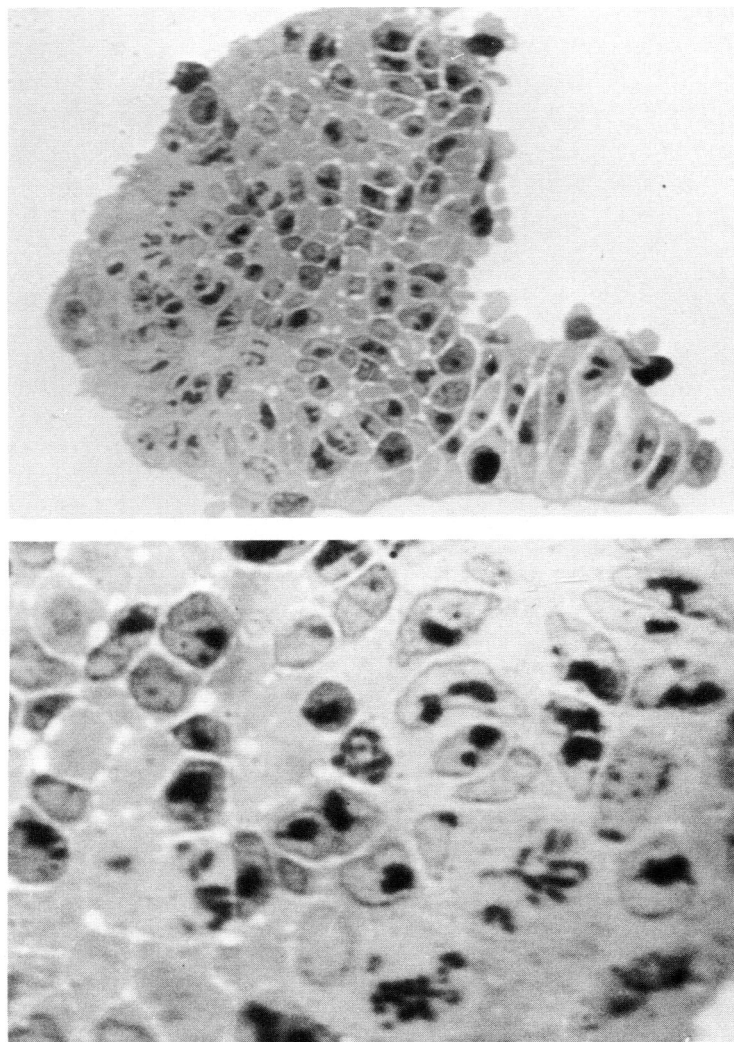
Fig 6. Ectoplacental cone core fragments after 72 h in suspension culture. Note absence of central necrosis and well-delineated nuclear membranes. 1 μm section. Toluidine blue (× 240) and detail (× 600).

1979), secreted matrices, or direct cell contact. Earlier studies have claimed that ectoplacental cone tissues can continue to grow in ectopic sites (Grobstein, 1950; Simmons & Russel, 1962; Billington, 1965; Clarke, 1969) and in non-pregnant uteri (Kirby & Cowell, 1969) in the absence of ICM derivatives. These investigations, however, did not discriminate between trophoblastic growth brought about by cellular hypertrophy and that due to hyperplasia.

The phenomenon of cell- and tissue-shape mediated growth control has been studied extensively *in vitro* and, for most of these studies, cells not destined to become giant have been used (Folkman & Moscona, 1978; Folkman & Green-

span, 1975). It has been found, however, that normal mouse mammary epithelial cultures will form diploid, uninucleate 'domes' when grown at high cell density but produce polyploid, multinucleate monolayers if cultured from sparsely seeded explants (Das, Hosick & Naudi, 1973). Such density-mediated changes in cell and tissue shape may alter the growth of diploid and polyploid tissues in a number of ways. For example, tissue compaction may limit cell stretching, a mechanical stress able to stimulate DNA synthesis *in vitro* (Curtis & Seehar, 1978) and possibly promote endoreduplication *in vivo* (Coulombre, Steinberg & Coulombre, 1963; Barlow & Sherman, 1972). On a subcellular level, such changes in cell and tissue shape may involve the redistribution of microfilaments (Bragina, Vasilev & Gelfand, 1974), alterations in surface adhesion (Marondas, 1973; Martz & Steinberg, 1974), junction formation (Ducibella & Anderson, 1975) and/or the modification of certain cell membrane components (Whittenberger & Glaser, 1972, 1978). Diffusion, either within dense monolayer cultures or compact tissue fragments may also be limiting for growth (Bissel, Farson & Tung, 1972; Holley, 1975; Folkman & Greenspan, 1975; Stoker, 1973; Dulbecco & Elkington, 1973; Kruse & Miedema, 1965; Leighton & Tchao, 1975). In cases where this is so, cultures of 'nutrient-deprived' cells usually become arrested in G_1 (Zetterberg & Auer, 1970; Tobey & Ley, 1970; Holley & Kiernan, 1974) but the trophoblastic tissues grown in the present study, in contrast to earlier investigations, were found to contain considerable numbers of [³H]-thymidine-labelled nuclei.

Although various pieces of direct (Zybina, 1970; Zybina & Tikhomirova, 1963; Zybina & Grischenko, 1970; Ansell & Snow, 1974) and indirect (Sherman, McLaren & Walker, 1972; Chapman, Ansell & McLaren, 1972; Gearhart & Mintz, 1972) evidence suggests that exceedingly large, e.g. $> 64\bar{c}$, trophoblastic nuclei grow via endomitotic and polytenic cycles, the mechanism(s) by which these giant cells initially acquire DNA contents greater than $4\bar{c}$ is not known. The finding of metaphases which contain multiples of the diploid number of chromosomes arranged either randomly or in pairs as well as occasional binucleate cells suggests that at least some trophoblastic tissues initially become giant via an acytokinetic mitosis and/or by endomitosis. Although polyploid trophoblastic metaphases are rarely found *in vivo* (Zybina & Grischenko, 1970), this is probably due to the large amount of time these cells spend in either (endo) G or (endo) S as compared with (endo) mitosis (reviewed by Nagl, 1978). Cells with large numbers of chromosomes have been found in other mammalian tissues known to contain giant nuclei (Biesele, 1944; Walker, 1958), different-sized mitotic figures have been recognized in membranous chorion (Wimsatt, 1951), and large multipolar trophoblastic mitoses have been seen in explants of rabbit blastocysts (Maximow, 1925). Diplochromosomes, which are induced at low frequencies in animal tissues (Deyson, 1966) by a brief exposure to colcemid (E. P. Evans, personal communication), or by prolonged colcemid treatment (Harris, 1971; Herreros, Guerro & Koma, 1966) and never found in cultures of

embryonic ectoderm, have been noted, on rare occasions, within 7·0-day mouse ectoplacental cone (M. Burtenshaw, unpublished). The syncitia found in the present study were not examined electronmicroscopically and could conceivably be composed of several, smaller multinucleates. However, since their outlines are, in most cases, easily traced and their peripheral contours smooth, it is most unlikely that they are merely masses of single cells. Therefore, it is suggested that cells derived solely from trophectoderm (Gardner & Papaioannou, 1975) are able to form multinucleates of different sizes *in vitro*. Thus, the syncitial differentiation of trophoblast apparently does not depend on either the presence of allantoic mesoderm (as proposed by Carpenter and Hernandez-Verdun (1974, 1975) or contact with other mesenchymal tissues (Glenister, 1965). Other 'trophoblastic' cultures have also been reported to contain multinucleate cells but the tissues from which these were originally derived were probably contaminated with foetal materials (Schlesinger & Koren, 1967; Koren & Behrman, 1968).

The postimplantation mouse embryo displays an exceedingly complex spatial distribution of rates of cellular growth and division. This complexity virtually precludes an experimental analysis of those control mechanisms which underly the growth of postimplantation trophoblast *in vivo*. However, by using an *in vitro* approach to this problem, it has been shown that the proliferation of postimplantation mouse trophoblast depends, in part, on a homotypic cellular interaction. Although the basis of this interaction is not known, it is able to suppress the tendency of diploid trophoblastic cells to undergo either endomitosis and/or acytokinetic mitosis once they have lost contact with their dividing neighbours.

REFERENCES

ANSELL, J. D. & SNOW, M. H. L. (1974). The chromosomes of giant trophoblast cells of the mouse. *Proc. R. Soc. Lond.* B **187**, 93–98.

BAILEY, N. T. J. (1973). *Statistical Methods in Biology*. London: The English Universities Press.

BARLOW, P. W. & SHERMAN, M. I. (1972). The biochemistry of differentiation of mouse trophoblast: Studies on polyploidy. *J. Embryol. exp. Morph.* **27**, 447–465.

BERNSTEIN, E. G., HOOPER, M. L., GRANDECHAMP, S. & EPHRUSSI, B. (1973). Alkaline phosphatase activity in mouse teratomas. *Proc. natn Acad. Sci., U.S.A.* **70**, 3899–3903.

BIESELE, J. (1944). Chromosome complexity in regenerating rat liver. *Cancer Res.* **4**, 232–235.

BILLINGTON, W. D. (1965). The invasiveness of transplanted mouse trophoblast and the influence of immunological factors. *J. Rep. Fert.* **10**, 343–352.

BISSEL, M. J., FARSON, D. & TUNG, A. S. C. (1977). Cell shape and hexose transport in normal and virus transformed cells in culture. *J. Supramolec. Struc.* **6**, 1–12.

BRAGINA, E., VASILEV, J. & GELFAND, J. M. (1976). Formation of bundles of microfilaments during spreading of fibroblasts on the substrate. *Expl Cell Res.* **97**, 241–248.

CARPENTER, S. J. (1972). Light and electronmicroscopic observations on the morphogenesis of the chorioallantoic placenta of the golden hamster. *Am. J. Anat.* **135**, 445–476.

CHAPMAN, V., ANSELL, J. D. & McLAREN, A. (1972). Trophoblastic giant cell differentiation in the mouse: Expression of glucose phosphate isomerase (GPI-1) electrophoretic variants in transferred and chimaeric embryos. *Devl Biol.* **29**, 48–54.

CLARKE, A. G. (1969). Factors affecting the growth of trophoblast transplanted to the testis. *J. Rep. Fert.* **18**, 539–541.

COPP, A. J. (1978). Interaction between inner cell mass and tropectoderm of the mouse blastocyst. I. A study of cellular proliferation. *J. Embryol. exp. Morph.* **48**, 109–125.

COPP, A. J. (1979). Interaction between inner cell mass and trophectoderm of the mouse blastocyst. II. The fate of polar trophectoderm. *J. Embryol. exp. Morph.* **51**, 109–120.

COULOMBRE, A. J., STEINBERG, S. J. & COULOMBRE, J. L. (1963). The role of intraocular pressure in the development of the chick eye: V. *Pigmented epithelium Invest. Opthal.* **2**, 83–89.

CURTIS, A. & SEEHAR, G. M. (1978). The control of cell division by tension or diffusion. *Nature, Lond.* **274**, 52–53.

DAS, N. K., HOSICK, K. L. & NANDI, S. (1974). Influence of seeding density on multicellular organization and nuclear events in cultures of normal and neoplastic mouse mammary epithelium. *J. natn. Cancer Inst.* **52**, 849–855.

DEYSON, G. (1968). Antimitotic substances. *Int. Rev. Cytol.* **24**, 99–148.

DIACUMAKOS, E. G. (1973). Methods for micromanipulation of human somatic cells in culture. In *Methods in Cell Biology*, vol. 8 (ed. D. M. Prescott), pp. 287–311, Academic Press: New York.

DUCIBELLA, T. & ANDERSON, E. (1975). Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. *Devl Biol.* **47**, 45–58.

DULBECCO, R. & ELKINGTON, J. (1973). Conditions limiting the multiplication of fibroblastic and epithelial cells in dense cultures. *Nature, Lond.* **246**, 197–199.

EVANS, E. P., BURTENSHAW, M. B. & FORD, C. E. (1972). Chromosomes of mouse embryos and newborn young: preparations from membranes and tail tips. *Stain Technol.* **47**, 229–234.

FOLKMAN, J. & GREENSPAN, H. (1975). Influence of geometry on the control of cell growth. *Biochim. biophys. Acta* **417**, 211–236.

FOLKMAN, J. & MOSCONA, A. (1978). Role of cell shape in growth control. *Nature, Lond.* **273**, 343–349.

GARDNER, R. L. (1972). An investigation of inner cell mass and trophoblastic tissue following their isolation from the mouse. *J. Embryol. exp. Morph.* **28**, 279–312.

GARDNER, R. L. & PAPAIOANNOU, V. E. (1975). Differentiation in the trophectoderm and inner cell mass. In *The Early Development of Mammals: Second Symposium of the British Society for Developmental Biology* (ed. M. Balls & A. E. Wild), pp. 107–132. Cambridge University Press.

GARDNER, R. L., PAPAIOANNOU, V. E. & BARTON, S. C. (1973). Origin of the ectoplacental cone and secondary giant cells in mouse blastocysts reconstituted from trophoblast and inner cell mass. *J. Embryol. exp. Morph.* **30**, 561–572.

GEARHART, J. D. & MINTZ, B. (1972). GPI subunit reassociation tests for maternal-foetal and foetal-foetal cell fusion in the mouse placenta. *Devl Biol.* **29**, 55–64.

GLENISTER, T. W. (1965). The behaviour of trophoblast when blastocysts effect nidation in organ culture. In *The Early Conceptus, Normal and Abnormal*, pp. 24–26. University of St Andrews, Dundee.

GROBSTEIN, C. (1950). Production of interocular hemorrhage by mouse trophoblast. *J. exp. Zool.* **114**, 359–373.

HARRIS, M. (1971). Polyploid series of mammalian cells. *Expl Cell Res.* **66**, 329–336.

HAYTHORN, S. R. (1928). Multinucleated giant cells with particular reference to the foreign body giant cell. *Archs Path.* **7**, 651–713.

HERNANDEZ-VERDUN, D. (1974). Morphogenesis of the syncitium of the mouse placenta: An ultrastructural study. *Cell Tis. Res.* **148**, 381–396.

HERNANDEZ-VERDUN, D. (1975). *In vitro* study of chorionic and ectoplacental differentiation in the mouse. *J. Embryol. exp. Morph.* **34**, 633–644.

HERREROS, B., GUERRO, A. & KOMA, E. (1966). Colcemid-induced polyploidy. *Lancet* ii, 500–501.

HOLLEY, R. (1975). Control of growth of mammalian cells in culture. *Nature, Lond.* **258**, 487–490.

HOLLEY, R. & KIERNAN, J. (1974). Control of the initiation of DNA synthesis in 3T3 cells: low molecular weight nutrients. *Proc. natn. Acad. Sci., U.S.A.* **71**, 2942–2945.

HSU, Y. C. (1980). Embryo growth and differentiation factors in embryonic sera of mammals. *Devl Biol.* **76**, 465–474.

KIRBY, D. R. (1963). The development of the mouse blastocyst transplanted to the crypt-orchid and scrotal testis. *J. Anat. (Lond.)* **97**, 119–130.

KIRBY, D. R. & COWELL, T. P. (1968). Trophoblast–host interactions. In *Epithelial–Mesenchymal Interactions in Carcinogenesis* (ed. R. Fleishmajer and R. E. Billingham), pp. 64–77. Baltimore; Williams and Wilkins.

KOREN, F. & BEHRMAN, S. J. (1968). Organ culture of pure mouse trophoblast. *Am. J. Obstet. Gynec.* **100**, 576–581.

KRUSE, P. & MIEDEMA, G. (1965). Production and characterization of multiple-layered populations of animal cells. *J. Cell Biol.* **27**, 273–279.

LEIGHTON, J. & TCHAO, R. (1975). Metabolic gradients in tissue culture studies of human cancer cells. In *Human Tumor Cells* in vitro (ed. J. Fogh), pp. 241–266. New York: Plenum.

LEY, K. D. & TOBEY, R. A. (1970). Regulation of initiation of DNA synthesis in Chinese hamster cells. II. Induction of DNA synthesis and cell division by isoleucine and glutamine in G_1-arrested cells in suspension culture. *J. Cell Biol.* **47**, 453–459.

MARONDAS, N. G. (1973). Chemical and mechanical requirements for fibroblast adhesion. *Nature, Lond.* **244**, 353–355.

MARTZ, E. & STEINBERG, M. S. (1974). Movement in a confluent 3T3 monolayer and the causes of contact inhibition of overlapping. *J. Cell Sci.* **15**, 201–216.

MAXIMOW, A. (1925). Tissue culture of young mammalian embryos. *Contr. Embryol. (Carnegie)* **25**, 236–300.

NAGL, W. (1978). *Endopolyploidy and Polyteny in Evolution and Development*. North Holland: Amsterdam.

PATTERSON, H. F. (1979). *In vivo* and *in vitro* studies on the early embryonic lethal oligo-syndactylism (Os) in the mouse. *J. Embryol. exp. Morph.* **52**, 115–125.

ROSSANT, J. & OFFER L. (1977). Properties of extraembryonic ectoderm isolated from post-implantation mouse embryos. *J. Embryol. exp. Morph.* **39**, 183–194.

SCHLESINGER, M. & KOREN, F. (1967). Mouse trophoblastic cells in tissue culture. *Fert. Steril.* **18**, 95–101.

SELLENS, M. H. & SHERMAN, M. I. (1980). Effects of culture conditions on the developmental programme of mouse blastocysts. *J. Embryol. exp. Morph.* **56**, 1–22. (Changes in media composition produce variation in nuclear DNA content.)

SHERMAN, M. I., MCLAREN, A. & WALKER, P. M. B. (1972). Mechanism of accumulation of DNA in giant cells of mouse trophoblast. *Nature New Biol.* **238**, 175–176.

SIEGEL, S. (1956). *Nonparametric Statistics for the Behavioural Sciences* (ed. S. Siegel), pp. 96–104. N.Y.: McGraw-Hill.

SIMMONS, R. L. & RUSSEL, P. S. (1962). Antigenicity of mouse trophoblast. *Annls N.Y. Acad. Sci.* **99**, 717–732.

SNEDECOR, G. W. & COCHRAN, W. G. (1976). *Statistical Methods*, 6th ed., p. 217 (formula 8.10.3). Iowa State University Press.

SNELL, G. D. & STEVENS, L. C. (1966). Early embryology. In *Biology of the Laboratory Mouse*, 2nd ed. (ed. E. L. Green), pp. 205–245. New York: McGraw-Hill.

STOKER, M. G. P. (1973). Role of diffusion boundary layer in contact inhibition of growth. *Nature, Lond.* **246**, 200–203.

TOBEY, R. & LEY, K. D. (1970). Regulation of initiation of DNA synthesis in chinese hamster cells. I. Production of stable, reversible G_1-arrested populations in suspension culture. *J. Cell Biol.* **46**, 151–157.

WALKER, B. E. (1958). Polyploidy and differentiation in the transitional epithelium of the mouse urinary bladder. *Chromosoma* **9**, 105–118.

WHITTENBERGER, B. & GLASER, L. (1977). Cell membrane fraction able to replace saturation density requirement. *Proc. natn Acad. Sci. U.S.A.* **74**, 2251–2255.

WHITTENBERGER, B. & GLASER, L. (1978). Cell saturation density is not determined by a diffusion-limited process. *Nature, Lond.* **272**, 821–822.

WIMSATT, W. A. (1951). Observations on the morphogenesis, cytochemistry and significance of the binucleate giant cells of the placenta and ruminants. *Am. J. Anat.* **89**, 233–282.

ZETTERBERG, A. & AUER, G. (1970). Proliferative activity and cytochemical properties of nuclear chromatin related to local cell density of epithelial cells. *Expl Cell Res.* **62**, 262–270.

ZYBINA, E. V. & TIKHOMIROVA, M. (1963). Endomitotic polyploidization of giant trophoblast cells. In *Cell Morphology and Cytochemistry*, pp. 53–63. Moscow: Leningrad.

ZYBINA, E. V. (1970). Special features of the polyploidization of trophoblast cells. *Tsitologiya* **12**, 1081–1094.

ZYBINA, E. V. & GRISCHENKO, T. H. (1970). Polyploid cells of the trophoblast indifferent regions of the white rat placenta. *Tsitologiya* **12**, 585–595.